PATENT

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# APPLICATION FOR UNITED STATES LETTERS PATENT

for

# SHORT BIOACTIVE PEPTIDES

by

*Donald R. Owen*

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## FIELD OF THE INVENTION

The invention relates to short length peptides containing phenylalanine, leucine, alanine, and lysine amino acid residues (F, L, A, and K; "FLAK peptides") in their primary sequence. In particular, FLAK peptides having desirable antimicrobial, antifungal, anticancer, and other biological activities are disclosed.

## BACKGROUND OF THE INVENTION

Various bioactive peptides have been reported in both the scientific literature and in issued patents. Peptides historically have been isolated from natural sources, and have recently been the subject of structure-function relationship studies. Additionally, natural peptides have served as starting points for the design of synthetic peptide analogs.

A review of peptide antibiotics was published by R.E.W. Hancock in 1997 (*Lancet* 349: 418-422). The structure, function, and clinical applications of various classes of peptides were discussed. An additional review of cationic peptide antibiotics was published in 1998 (Hancock, R.E.W. and Lehrer, R. *Trends Biotechnol.* 16: 82-88). The peptides are typically cationic amphipathic molecules of 12 to 45 amino acids in length. The peptides permeabilize cell membranes leading to the control of microbial agents. The clinical potential of host defense cationic peptides was discussed by R.E.W. Hancock in 1999 (*Drugs* 57(4): 469-473; *Antimicrobial Agents and Chemotherapy* 43(6): 1317-1323). The antibacterial, antifungal, antiviral, anticancer, and wound healing properties of the class of peptides are discussed.

Reviews of the structural features of helical antimicrobial peptides, and their presumed mechanisms of action have been published (see, for example, Dathe, M. and Wieprecht, T. *Biochimica et Biophysica Acta* 1462: 71-87 (1999); Epand, R.M. and Vogel H.J. *Biochimica et Biophysica Acta* 1462: 11-28 (1999)). Structural parameters believed to be capable of modulating activity and selectivity include helicity, hydrophobic moment, hydrophobicity, angle subtended by the hydrophilic/hydrophobic helix surfaces, and charge.

A wide array of naturally occurring alpha helical peptides have been reported. The following are representative of the many references in the field.

Cecropins are a family of α-helical peptides isolated from insects. Cecropins are known for their antibacterial properties, as described in U.S. Patent Nos. 4,355,104 and 4,520,016. The cecropins were generally found to have activity against gram-negative bacteria, but not against all gram-negative bacteria. Cecropins were found not to have activity against eucaryotic cells (Andreu, et al., *Biochemistry* 24: 163-188 (1985); Boman, et al., *Developmental and Comparative Immunol.* 9: 551-558 (1985); Steiner et al., *Nature* 292: 246-248 (1981)). Cecropins from *Drosophila* and *Hyalphora* were presented as having activity against various strains of fungi (Ekengren, S. and Hultmark, D., *Insect Biochem. and Molec. Biol.* 29: 965-972 (1999)). Cecropin A from mosquito *Aedes aegypti* is reportedly different from most insect cecropins in that it lacks tryptophan and C-terminal amidation (Lowenberger, C. et al., *J. Biol. Chem.* 274(29): 20092-20097 (1999)).

Frogs from the genus *Rana* produce a wide array of antimicrobial peptides in their skin (Goraya, J. et al., *Eur. J. Biochem.* 267: 894-900 (2000)). Peptides as short as 13 amino acids were reported, and were grouped into structural families. The sequences showed little or no sequence identity to peptides isolated from frogs of other genera, such as the magainin and dermaseptin peptides.

U.S. Patent No. 5,962,410 disclosed the inhibition of eucaryotic pathogens, and the stimulation of lymphocytes and fibroblasts with lytic peptides such as cecropins and sarcotoxins. Various peptides presented include Cecropin B, Cecropin SB-37, Cecropin A, Cecropin D, Shiva-1, Lepidopteran, Sarcotoxin 1A, Sarcotoxin 1B, and Sarcotoxin 1C.

Transgenic mice producing the Shiva-1 cecropin class lytic peptide were reported by Reed, W.A. et al., *Transgenic Res.* 6: 337-347 (1997). Infection of the transgenic mice with a *Brucella abortus* challenge resulted in a reduction of the number of bacteria relative to infection of non-transgenic mice.

Magainin is an α-helical 23 amino acid peptide isolated from the skin of the African frog *Xenopus laevis* (Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* 84: 5449-5453 (1987).

Cathelin associated α-helical peptides of 23 to 38 amino acids are found in the blood cells of sheep, humans, cattle, pigs, mice, and rabbits (Zanetti, M. et al., *FEBS Lett.* 374: 1-5 (1995)).

The antimicrobial activities of buforin II, cecropin P1, indolicidin, magainin II, nisin, and ranalexin were reported by Giacomette, A. et al. (*Peptides* 20: 1265-1273 (1999)). The peptides showed variable activities against bacteria and yeast.

Various synthetic peptides have been prepared and assayed both *in vitro* and *in vivo*.

U.S. Patent No. 5,861,478 disclosed synthetic lytic peptides of about 20 to 40 amino acids which adopt an α-helical conformation. The peptides are effective in the treatment of microbial infections, wounds, and cancer. The peptides disclosed include cecropin B, SB-37*, LSB-37, SB-37, Shiva 1 and 10-12, β-fibrin signal peptide, Manitou 1-2, Hecate 1-3, Anubis 1-5 and 8, and Vishnu 1-3 and 8.

Hecate was described as a synthetic peptide analog of melittin by Baghian, A. et al. (*Peptides* 18(2): 177-183 (1997)). The peptides differ in their charge distribution, but not in their amphipathic alpha helical conformation. Hecate inhibited herpes simplex virus (HSV-1) while not adversely affecting cell growth and protein synthesis.

Synthetic peptides D2A21, D4E1, D2A22, D5C, D5C1, D4E, and D4B were described in Schwab, U. et al., *Antimicrob. Agents and Chemotherapy* 43(6): 1435-1440 (1999). Activities against various bacterial strains were presented.

Hybrid peptides made of cecropin and melittin peptides were reportedly prepared and assayed by Juvvadi, P. et al. (*J. Peptide Res.* 53: 244-251 (1999)). Hybrids were synthesized to investigate the effects of sequence, amide bond direction (helix dipole), charge, amphipathicity, and hydrophobicity on channel forming ability and on antibacterial activity. Sequence and amide bond direction were suggested to be important structural requirements for the activity of the hybrids.

A 26 amino acid insect cecropin - bee melittin hybrid, and analogs thereof, were described in a study of salt resistance (Friedrich, C. et al., *Antimicrobial Agents and Chemotherapy* 43(7): 1542-1548 (1999)). A tryptophan residue in the second position was found to be critical for activity. Modest changes in sequence were found to lead to substantial changes in the properties of the peptides.

The effects of proline residues on the antibacterial properties of α-helical peptides has been published (Zhang, L. et al., *Biochem.* 38: 8102-8111 (1999)). The addition of prolines was reported to change the membrane insertion properties, and the replacement of a single proline may change an antimicrobial peptide into a toxin.

A series of peptides having between 18 and 30 amino acids were prepared in order to test the effects of changes in sequence and charge on antibacterial properties (Scott, M.G., et al., *Infect. Immun.* 67(4): 2005-2009 (1999)). No significant correlation was found between length, charge, or hydrophobicity and the antimicrobial activity of the peptides. A general trend was found that shorter peptides were less active than longer peptides, although the authors expressed that this effect would probably be sequence dependent.

"Modellins", a group of synthetic peptides were prepared and assayed to compare sequence and structure relationships (Bessalle, R. et al. *J. Med. Chem.* 36: 1203-1209 (1993)). Peptides of 16 and 17 amino acids having hydrophobic and hydrophilic opposite faces were highly hemolytic and antibacterial. Smaller peptides tended to have lower biological activities.

A cecropin-melittin hybrid peptide and an amidated flounder peptide were found to protect salmon from *Vibrio anguillarum* infections *in vivo* (Jia, X. et al., *Appl. Environ. Microbiol.* 66(5): 1928-1932 (2000)). Osmotic pumps were used to deliver a continuous dose of either peptide to the fish.

Amphipathic peptides have been reported as being capable of enhancing wound healing and stimulating fibroblast and keratinocyte growth *in vivo* (U.S. Patent Nos. 6,001,805 and 5,561,107). Transgenic plants have been reportedly prepared expressing lytic peptides as a fusion protein with ubiquitin (U.S. Patent No. 6,084,156). Methylated lysine rich lytic peptides were reportedly prepared, displaying improved proteolytic resistance (U.S. Patent No. 5,717,064).

While a number of natural and synthetic peptides exist, there exists a need for improved bioactive peptides and methods for their use.

## SUMMARY OF THE INVENTION

Short (i.e. no more than 23 amino acids in length) peptides containing phenylalanine, leucine, alanine, and lysine amino acid residues in their primary sequence are disclosed. The peptides display desirable antibacterial, antifungal, anticancer biological activities, and also cause stimulation and proliferation of human fibroblasts and lymphocytes.

## DESCRIPTION OF THE SEQUENCE LISTINGS

The following sequence listings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these sequences in combination with the detailed description of specific embodiments presented herein.

Table 1

| SEQ ID NO: | Name | P-No. | Primary sequence |
|---|---|---|---|
| 1 | Hecate AC #1010 | 1 | FALALKALKKALKKLKKALKKAL-COOH |
| 2 | Hecate AM | 2 | FALALKALKKALKKLKKALKKAL-NH2 |
| 3 | SB-37 AC #1018 | 5 | MPKWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG-COOH |
| 4 | Shiva 10 AM | 11 | FAKKLAKKLKKLAKKLAKLALAL-NH2 |
| 5 | SB-37 AM | 12 | MPKWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG-NH2 |
| 6 | Shiva 10 AC #1015 | 13 | FAKKLAKKLKKLAKKLAKLALAL-COOH |
| 7 | Magainin 2 | 16 | GIGKFLHSAKKFGKAFVGGIMNS-NH2 |
| 8 | FLAK01 AM | 23 | FALAAKALKKLAKKLKKLAKKAL-NH2 |
| 9 | FLAK03 AM | 24 | FALALKALKKLLKKLKKLAKKAL-NH2 |
| 10 | FLAK04 AM | 25 | FALALKALKKLAKKLKKLAKKAL-NH2 |
| 11 | FLAK05 AM | 26 | FALAKLAKKAKAKLKKALKAL-NH2 |
| 12 | FLAK06 AM | 27 | FALALKALKKLKKALKKAL-NH2 |
| 13 | FLAK06 AC | 27 B | FALALKALKKLKKALKKAL-COOH |
| 14 | FLAK06 R-AC | 27 C | FAKKLAKKLKKLAKLALAL-COOH |
| 15 | KAL V | 30 | VALALKALKKALKKLKKALKKAL-NH2 |
| 16 | FLAK 17 AM | 34 | FALALKKALKALKKAL-NH2 |
| 17 | FLAK 26 AM | 35 | FAKKLAKLAKKLAKLAL-NH2 |
| 18 | FLAK 25 AM | 36 | FAKKLAKLAKKLAKLALAL-NH2 |
| 19 | Hecate 2DAc | 37 | FALALKALKKAL-(D)-K-(D)-KLKKALKKAL-COOH |
| 20 | FLAK43 AM | 38 | FAKKLAKLAKKLLAL-NH2 |
| 21 | FLAK44 AM | 39 | FAKKLAKLAKKALAL-NH2 |
| 22 | FLAK62 AM | 40 | FALAKKALKKAKKAL-NH2 |

| | | | |
|---|---|---|---|
| 23 | FLAK 06R-AM | 41 | FAKKLAKKLKKLAKLALAK-NH2 |
| 24 | MSI-78 AM | 42 | GIGKFLKKAKKFGKAFVKILKK-NH2 |
| 25 | FLAK50 | 43 | FAKLLAKLAKKLL-NH2 |
| 26 | FLAK51 | 44 | FAKKLAKLALKLAKL-NH2 |
| 27 | FLAK57 | 45 | FAKKLAKKLAKLAL-NH2 |
| 28 | FLAK71 | 46 | FAKKLKKLAKLAKKL-NH2 |
| 29 | FLAK77 | 47 | FAKKALKALKKL-NH2 |
| 30 | FLAK50V | 48 | VAKLLAKLAKKLL-NH2 |
| 31 | FLAK50F | 49 | FAKLLAKLAKKL-NH2 |
| 32 | FLAK26V AM | 50 | VAKKLAKLAKKLAKLAL-NH2 |
| 33 | CAME-15 | 53 | KWKLFKKIGAVLKVL-NH2 |
| 34 | FLAK50C | 54 | FAKLLAKLAKKAL-NH2 |
| 35 | FLAK50D | 55 | FAKLLAKALKKLL-NH2 |
| 36 | FLAK 50E | 56 | FAKLLKLAAKKLL-NH2 |
| 37 | FLAK80 | 57 | FAKLLAKKLL-NH2 |
| 38 | FLAK81 | 58 | FAKKLAKALL-NH2 |
| 39 | FLAK82 | 59 | FAKKLAKKLL-NH2 |
| 40 | FLAK83M | 60 | FAKLAKKLL-NH2 |
| 41 | FLAK 26 Ac | 61 | FAKKLAKLAKKLAKLAL-COOH |
| 42 | Indolicidin | 63 | ILPWKWPWWPWRR-NH2 |
| 43 | FLAK 17C | 64 | FAKALKALLKALKAL-NH2 |
| 44 | FLAK 50H | 65 | FAKLLAKLAKAKL-NH2 |
| 45 | FLAK 50G | 66 | FAKLLAKLAKLKL-NH2 |
| 46 | Shiva Deriv P69+KWKL | 70 | FAKKLAKKLKKLAKKLAKKWKL-NH2 |
| 47 | Shiva 10 (1-18 AC) | 71 | FAKKLAKKLKKLAKKLAK-COOH |
| 48 | Shiva 10 peptide 71+KWKL | 72 | FAKKLAKKLKKLAKKLAKKWKL-COOH |
| 49 | CA(1-7)Shiva10(1-16) | 73 | KWKLFKKKTKLFKKFAKKLAKKL-NH2 |
| 50 | FLAK 54 | 74 | FAKKLAKKLAKAL-NH2 |
| 51 | FLAK 56 | 75 | FAKKLAKKLAKLL-NH2 |
| 52 | FLAK 58 | 76 | FAKKLAKKLAKAAL-NH2 |
| 53 | FLAK 72 | 77 | FAKKLAKKAKLAKKL-NH2 |
| 54 | FLAK 75 | 79 | FAKKLKKLAKKL-NH2 |
| 55 | Shiva 10 (1-16) Ac | 80 | KTKLFKKFAKKLAKKLKKLAKKL-COOH |
| 56 | CA(1-7)Shiva10 (1-16)-COOH | 81 | KWKLFKKKTKLFKKFAKKLAKKL-COOH |
| 57 | Indolocidin-ac | 91 | ILPWKWPWWPWRR-COOH |
| 58 | FLAK50B | 92 | FAKALAKLAKKLL-NH2 |
| 59 | FLAK50J | 93 | FAKLLAKLAKKAA-NH2 |
| 60 | FLAK50I | 94 | FAKLLALALKLKL-NH2 |
| 61 | FLAK50K | 95 | FAKLLAKLAKAKA-NH2 |
| 62 | FLAK50L | 96 | FAKLLAKLAKAKG-NH2 |
| 63 | Shiva-11 | 98 | FAKKLAKKLKKLAKKLAKLALALKALALKAL-NH2 |
| 64 | Shiva 11 [(1-16)ME(2-9] - COOH | 99 | FAKKLAKKLKKLAKKLIGAVLKV-COOH |
| 65 | FLAK 50N | 101 | FAKLLAKALKLKL-NH2 |
| 66 | FLAK 50O | 102 | FAKLLAKALKKAL-NH2 |
| 67 | FLAK 50P | 103 | FAKLLAKALKKL-NH2 |
| 68 | CA(1-&Hecate(11/23) | 104 | KWKLFKKALKKLKKALKKAL-NH2 |

| 69 | PYL-ME | 105 | KIAKVALAKLGIGAVLKVLTTGL-NH2 |
|---|---|---|---|
| 70 | FLAG26-D1 | 106 | FAKKLAKLAKKL-NH2 |
| 71 | Vishnu3 | 107 | MPKEKVFLKIEKMGRNIRN-NH2 |
| 72 | Melittin | 108 | GIGAVLKVLTTGLPALISWIKRKRQQ-NH2 |
| 73 | FLAK26-D2 | 109 | FAKKLAKLAKKLAKAL-NH2 |
| 74 | FLAG26-D3 | 110 | FAKKLLAKALKL-NH2 |
| 75 | FLAK50 Q1 | 111 | FAKFLAKFLKKAL-NH2 |
| 76 | FLAK50 Q2 | 112 | FAKLLFKALKKAL-NH2 |
| 77 | FLAK50 Q3 | 113 | FAKLLAKFLKKAL-NH2 |
| 78 | FLAK50 Q4 | 114 | FAKLLAKAFKKAL-NH2 |
| 79 | FLAK50 Q5 | 117 | FAKLFAKAFKKAL-NH2 |
| 80 | FLAK50 Q6 | 118 | FAKLLAKALKKFL-NH2 |
| 81 | FLAK50 Q7 | 119 | FAKLLAKALKKFAL-NH2 |
| 82 | FLAK50 Q8 | 120 | FAKLLAKLAKKFAL-NH2 |
| 83 | FLAK50 Q9 | 121 | FAKLFAKLAKKFAL-NH2 |
| 84 | FLAK50 Q10 | 122 | FKLAFKLAKKAFL-NH2 |
| 85 | FLAK50 T1 | 123 | FAKLLAKLAK-NH2 |
| 86 | FLAK50 T2 | 124 | FAKLLAKLAKKVL-NH2 |
| 87 | FLAK50 T3 | 125 | FAKLLAKLAKKIL-NH2 |
| 88 | FLAK50 T4 | 126 | FAKLLAKLAKKEL-NH2 |
| 89 | FLAK50 T5 | 127 | FAKLLAKLAKKSL-NH2 |
| 90 | FLAK90 | 128 | FAKLA-NH2 |
| 91 | FLAK91 | 129 | FAKLF-NH2 |
| 92 | FLAK92 | 130 | KAKLF-NH2 |
| 93 | FLAK93 | 131 | KWKLF-NH2 |
| 94 | FLAK50 Z1 | 132 | FGKGIGKVGKKLL-NH2 |
| 95 | FLAK50 Z2 | 133 | FAFGKGIGKVGKKLL-NH2 |
| 96 | FLAK50 Z3 | 134 | FAKAIAKIAFGKGIGKVGKKLL-NH2 |
| 97 | FLAK50 Z4 | 135 | FAKLWAKLAFGKGIGKVGKKLL-NH2 |
| 98 | FLAK50 Z5 | 136 | FAKLWAKLAKKL-NH2 |
| 99 | FLAK50 Z6 | 137 | FAKGVGKVGKKAL-NH2 |
| 100 | FLAK50 Z7 | 138 | FAFGKGIGKIGKKGL-NH2 |
| 101 | FLAK50 Z8 | 139 | FAKIIAKIAKIAKKIL-NH2 |
| 102 | FLAK50 Z9 | 140 | FAFAKIIAKIAKKII-NH2 |
| 103 | FLAK94 | 141 | FALALKA-NH2 |
| 104 | FLAK93B | 142 | KWKLAKKALALL-NH2 |
| 105 | FLAK50 Z10 | 143 | FAKIIAKIAKKI-NH2 |
| 106 | FLAK96 | 144 | FALALKALKKAL-NH2 |
| 107 | FLAK97 | 145 | FALKALKK-NH2 |
| 108 | FLAK98 | 146 | KYKKALKKLAKLL-NH2 |
| 109 | FKRLA | 147 | FKRLAKIKVLRLAKIKR-NH2 |
| 110 | FLAK91B | 148 | FAKLAKKALAKLL-NH2 |
| 111 | FLAK92B | 149 | KAKLAKKALAKLL-NH2 |
| 112 | FLAK99 | 150 | KLALKLALKALKAAKLA-NH2 |
| 113 | FLAK50T6 | 151 | FAKLLAKLAKK-NH2 |
| 114 | FLAK50T7 | 152 | FAKLLAKLAKKGL-NH2 |
| 115 | FLAK95 | 153 | FALKALKKLKKALKKAL-NH2 |
| 116 | FLAK50T8 | 154 | VAKLLAKLAKKVL-NH2 |
| 117 | FLAK50T9 | 155 | YAKLLAKLAKKAL-NH2 |
| 118 | FLAK100-CO2H | 156 | KLLKLLLKLYKKLLKLL-COOH |
| 119 | FAGVL | 157 | FAVGLRAIKRALKKLRRGVRKVAKDL-NH2 |
| 120 | Modelin-5 | 159 | KLAKKLAKLAKLAKAL-NH2 |

| | | | |
|---|---|---|---|
| 121 | Modelin-5-CO2H | 160 | KLAKKLAKLAKLAKAL-COOH |
| 122 | Modelin-8 | 161 | KWKKLAKKW-NH2 |
| 123 | Modelin-8-CO2H | 162 | KWKKLAKKW-COOH |
| 124 | Modelin-1 | 163 | KLWKKWAKKWLKLWKAW-NH2 |
| 125 | Modelin-1-CO2H | 164 | KLWKKWAKKWLKLWKA-COOH |
| 126 | FLAK120 | 165 | FALALKALKKL-NH2 |
| 127 | FLAK121 | 166 | FALAKALKKAL-NH2 |
| 128 | FLAK96B | 167 | FALALKLAKKAL-NH2 |
| 129 | FLAK96G | 168 | FALLKL-NH2 |
| 130 | FLAK96F | 169 | FALALKALKK-NH2 |
| 131 | FLAK96C | 170 | FALKALKKAL-NH2 |
| 132 | FLAK96D | 171 | FALLKALKKAL-NH2 |
| 133 | Modelin-8B | 172 | KWKK-NH2 |
| 134 | Modelin-8C | 173 | KWKKL-NH2 |
| 135 | Modelin-8D | 174 | KFKKLAKKF-NH2 |
| 136 | Modelin-8E | 175 | KFKKLAKKW-NH2 |
| 137 | Flak 96 | 176 | FALALKALKKA-NH2 |
| 138 | Flak 96I | 177 | FALLKALLKKAL-NH2 |
| 139 | Flak 96J | 178 | FALALKLAKKL-NH2 |
| 140 | Flak 96L | 179 | LKKLAKLALAF-NH2 |
| 141 | FLAK-120G | 180 | VALALKALKKL-NH2 |
| 142 | FLAK-120D | 181 | FALALKLKKL-NH2 |
| 143 | FLAK-120C | 182 | FALALKAKKL-NH2 |
| 144 | FLAK-120B | 183 | FALA-NH2 |
| 145 | FLAK-120F | 184 | WALAL-NH2 |
| 146 | Magainin2wisc | 300 | GIGKFLHAAKKFAKAFVAEIMNS-NH2 |
| 147 | D2A21 | 301 | FAKKFAKKFKKFAKKFAKFAFAF-NH2 |
| 148 | KSL-1 | 302 | KKVVFKVKFK-NH2 |
| 149 | KSL-7 | 303 | FKVKFKVKVK-NH2 |
| 150 | LSB-37 | 306 | LPKWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG-NH2 |
| 151 | Anubis-2 | 307 | FAKKLAKKLKKLAKKLAKLAKKL-NH2 |
| 152 | FLAK17CV | 501 | VAKALKALLKALKAL-NH2 |
| 153 | FLAK50Q1V | 502 | VAKFLAKFLKKAL-NH2 |
| 154 | D2A21V | 503 | VAKKFAKKFKKFAKKFAKFAFAF-NH2 |
| 155 | FLAK25AMV | 504 | VAKKLAKLAKKLAKLALAL-NH2 |
| 156 | FLAK43AMV | 505 | VAKKLAKLAKKLLAL-NH2 |
| 157 | FLAK50DV | 506 | VAKLLAKALKKLL-NH2 |
| 158 | HECATE AMV | 507 | VALALKALKKALKKLKKALKKAL-NH2 |
| 159 | HECATE ACV | 508 | VALALKALKKALKKLKKALKKAL-COOH |
| 160 | FLAK04AMV | 509 | VALALKALKKLAKKLKKLAKKAL-NH2 |
| 161 | FLAK03AMV | 510 | VALALKALKKLLKKLKKLAKKAL-NH2 |
| 162 | D-Shiva 10 AC | 67 | (D)-FAKKLAKKLKKLAKKLAKLALAL-COOH |
| 163 | Shiva 11 AC | 100 | FAKKLAKKLKKLAKKLAKLALALKALALKA-COOH |
| 164 | Shiva 10 (1-18)AM | 69 | FAKKLAKKLKKLAKKLAK-NH2 |
| 165 | FLAK 50M | 97 | FAKLLALALKKAL-NH2 |

## DETAILED DESCRIPTION OF THE INVENTION

The invention is generally directed towards peptides having desirable biological properties, and their use. It is surprising that the peptides are efficacious due to their short length as compared to other peptides described in the art.

Peptides

One embodiment of the invention is directed towards an isolated peptide comprising phenylalanine, leucine, alanine, and lysine residues, wherein the peptide is about 5 to about 23 amino acids in length. The peptide can have a minimum length of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or about 18 amino acids. The peptide can have a maximum length of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or about 23 amino acids. The peptide can be about 5 to about 20 amino acids in length. The peptide can consist essentially of, or consist of phenylalanine, leucine, alanine, and lysine residues. The peptide can have a percent amino acid composition of phenylalanine, leucine, alanine, and lysine residues of at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%. The peptide can generally be any of the listed SEQ ID NOS which fall within these various guidelines, and more preferably is SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:71, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:77, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:106, SEQ ID NO:108, SEQ ID NO:112, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:126, SEQ ID NO:127, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:131, SEQ ID NO:132, SEQ ID NO:137, SEQ ID NO:138, SEQ ID

NO:139, SEQ ID NO:140, SEQ ID NO:141, SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:152, SEQ ID NO:159, SEQ ID NO:162, SEQ ID NO:163, SEQ ID NO:164, and SEQ ID NO:165. The peptide is preferably not hecate-1, anubis-1, anubis-2, anubis-5, anubis-8, vishnu-1, vishnu-2, vishnu-3, vishnu-8, or shiva-10.

The peptide can be similar to any of the above described peptides, and preferably is similar to SEQ ID NO:2 (or SEQ ID NO:16 or SEQ ID NO:126), SEQ ID NO:4 (or SEQ ID NO:14 or SEQ ID NO:17), SEQ ID NO:25, SEQ ID NO:43, SEQ ID NO:75, SEQ ID NO:84, SEQ ID NO:115, or SEQ ID NO:132 as determined by percent identity. The percent identity between the peptides is preferably at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%. Percent identity is determined using a sequence alignment by the commercial product CLUSTALW. The number of aligned amino acids are divided by the length of the shorter peptide, and the result is multiplied by 100% to determine percent identity. If the length of the shorter peptide is less than 10 amino acids, the number of aligned amino acids are divided by 10, and the result is multiplied by 100% to determine percent identity.

The peptides can comprise D- or L- amino acids. The peptides can comprise all D- amino acids. The peptides can have an acid C-terminus (-$CO_2H$) or an amide C-terminus (-$CONH_2$, -CONHR, or -$CONR_2$).

Methods of use

An additional embodiment of the invention is directed towards methods of using the above described peptides. The methods of use preferably do not cause injury or kill normal uninfected mammalian cells. The methods of use at therapeutic dose levels preferably do not cause injury to or kill normal uninfected or non-neoplastic mammalian cells. The methods of use may involve the use of a single peptide, or may involve the use of multiple peptides.

An embodiment of the invention is the use of the above described peptides to inhibit or kill microbial cells (microorganisms). The microorganisms may be bacterial cells, fungal cells, protozoa, viruses, or eucaryotic cells infected with pathogenic microorganisms. The method generally is directed towards the contacting of microorganisms with the peptide. The contacting step can be performed *in vivo*, *in vitro*,

topically, orally, transdermally, systemically, or by any other method known to those of skill in the art. The contacting step is preferably performed at a concentration sufficient to inhibit or kill the microorganisms. The concentration of the peptide can be at least about 0.1 μM, at least about 0.5 μM, at least about 1 μM, at least about 10 μM, at least about 20 μM, at least about 50 μM, or at least about 100 μM. The methods of use can be directed towards the inhibition or killing of microorganisms such as bacteria, gram positive bacteria, gram negative bacteria, mycobacteria, yeast, fungus, algae, protozoa, viruses, and intracellular organisms. Specific examples include, but are not limited to, *Staphylococcus*, *Staphylococcus aureus*, *Pseudomonas*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Chlamydia*, *Candida albicans*, *Saccharomyces*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Trypanosoma cruzi*, or *Plasmodium falciparum*. The contacting step can be performed by systemic injection, oral, subcutaneous, IP, IM, IV injection, or by topical application. For injection, the dosage can be between any of the following concentrations: about 1 mg/kg , about 5 mg/kg, about 10 mg/kg, about 25 mg/kg, about 50 mg/kg, about 75 mg/kg, and about 100 mg/kg. The contacting step can be performed on a mammal, a cat, a dog, a cow, a horse, a pig, a bird, a chicken, a plant, a fish, or a human.

Preferred peptides for antibacterial applications include SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:60, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:77, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:84, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:93, SEQ ID NO:106, SEQ ID NO:108, SEQ ID NO:112, SEQ ID NO:115, SEQ ID NO:126, SEQ ID NO:128, SEQ ID NO:162, SEQ ID NO:163, SEQ ID NO:164, and SEQ ID NO:165.

Preferred peptides for antifungal applications include SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:25, SEQ ID NO:30, SEQ ID NO:35, SEQ ID NO:58, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:106, SEQ ID NO:108, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:126, SEQ ID NO:128, SEQ ID NO:131, SEQ ID NO:143, SEQ ID NO:163, and SEQ ID NO:165.

An additional embodiment of the invention is the use of any of the above described peptides to inhibit or kill cancer cells. The method generally is directed towards the contacting of cancer cells with the peptide. The contacting step can be performed *in vivo*, *in vitro*, topically, orally, transdermally, systemically, or by any other method known to those of skill in the art. The contacting step is preferably performed at a concentration sufficient to inhibit or kill the cancer cells. The concentration of the peptide can be at least about at least about 0.1 μM, at least about 0.5 μM, at least about 1 μM, at least about 10 μM, at least about 20 μM, at least about 50 μM, or at least about 100 μM. The cancer cells can generally be any type of cancer cells. The cancer cells can be sarcomas, lymphomas, carcinomas, leukemias, breast cancer cells, colon cancer cells, skin cancer cells, ovarian cancer cells, cervical cancer cells, testicular cancer cells, lung cancer cells, prostate cancer cells, and skin cancer cells. The contacting step can be performed by subcutaneous, IP injection, IM injection, IV injection, direct tumor injection, or topical application. For injection, the dosage can be between any of the following concentrations: about 0.1 mg/kg, about 1 mg/kg , about 5 mg/kg, about 10 mg/kg, about 25 mg/kg, about 50 mg/kg, about 75 mg/kg, and about 100 mg/kg. The contacting step can be performed on a mammal, a cat, a dog, a cow, a horse, a pig, a bird, a chicken, a plant, a fish, a goat, a sheep, or a human. The inhibition of cancer cells can generally be any inhibition of growth of the cancer cells as compared to the cancer cells without peptide treatment. The inhibition is preferably at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, and ideally 100% inhibition of growth. The inhibition may be achieved by lysis of the cancer cells or by other means. The cancer inhibiting peptide can be used synergistically with other cancer chemotherapeutic agents.

Preferred peptides for anticancer applications include SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:35, SEQ ID NO:46, SEQ ID NO:51, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:60, SEQ ID NO:68, SEQ ID NO:75, SEQ ID NO:86, SEQ ID NO:152, and SEQ ID NO:162

An additional embodiment of the invention is directed towards a method for promoting the stimulation and/or proliferation of cells. The method can comprise contacting the cells and a composition, wherein the composition comprises a peptide. The peptide can be any of the above described peptides. The concentration of the peptide in the composition can be about 0.01 μM to about 500 μM, about 0.1 μM to about 100 μM, about 1 μM to about 50 μM, or about 1 μM to about 10 μM. The cells can generally be any type of cells, and preferably are mammalian cells, specifically including, but not limited to fibroblast and leukocyte cells, including lymphocyte and phagocytic cells. The metabolic stimulation and/or proliferation of the cells is preferably increased by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, or 200% relative to the same cells not contacted with the composition. The composition can further comprise a growth factor. The stimulatory and proliferative properties of some of the FLAK peptides hold promise for their application in skin care, wound healing, and in immunomodulation of compromised mammalian immune systems.

Preferred peptides for stimulation and proliferation applications include SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:20, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:71, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:77, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:87, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:126, SEQ ID NO:127, SEQ ID NO:129, SEQ ID NO:132, SEQ ID NO:137, SEQ ID NO:138, SEQ ID NO:139, SEQ ID

NO:140, SEQ ID NO:141, SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:159, SEQ ID NO:162, and SEQ ID NO:164.

An additional embodiment of the invention is directed towards a method for promoting wound healing of skin or ocular and internal body tissues damaged by normal aging, disease, injury, or by surgery or other medical procedures. The method can comprise administering to the wound of an animal a composition, wherein the composition comprises any of the above described peptides. The concentration of the peptide in the composition can be about 0.01 μM to about 500 μM, about 0.1 μM to about 100 μM, about 1 μM to about 50 μM, or about 1 μM to about 10 μM. The composition can be administered to the wound topically or by systemic delivery. The animal can generally be any kind of animal, preferably is a mammal, and more preferably is a human, cow, horse, cat, dog, pig, goat, or sheep. The promotion of wound healing is preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, or 200% relative to the same wound not contacted with the composition.

Preferred peptides for wound healing applications include SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:20, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:71, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:77, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:87, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:126, SEQ ID NO:127, SEQ ID NO:129, SEQ ID NO:132, SEQ ID NO:137, SEQ ID NO:138, SEQ ID NO:139, SEQ ID NO:140, SEQ ID NO:141, SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:159, SEQ ID NO:162, and SEQ ID NO:164.

A further embodiment of the invention is directed towards methods for the additive or synergistic enhancement of the activity of a therapeutic agent. The method can comprise preparing a composition, wherein the composition comprises a peptide and a therapeutic agent. Alternatively, the method may comprise co-therapy treatment with a

peptide (or peptides) used in conjunction with other therapeutic agents. The peptide can be any of the above described peptides. The therapeutic agent can generally be any therapeutic agent, and preferably is an antibiotic, an antimicrobial agent, a growth factor, a chemotherapy agent, an antimicrobial agent, lysozyme, a chelating agent, or EDTA. Preferably, the activity of the composition is higher than the activity of the same composition containing the therapeutic agent but lacking the peptide. The composition or co-therapy can be used in *in vitro*, *in vivo*, topical, oral, IV, IM, IP, and transdermal applications. The enhancement of the activity of the composition containing the therapeutic agent and the peptide is preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, or 200% relative to the activity of the therapeutic agent alone.

Generally, any peptide which is active on a stand-alone basis against a target is preferred for use to increase either additively or synergistically the activity of another therapeutic agent against that target. If several peptides are candidates for a given synergy application, then the less toxic peptides would be more favorably considered.

The following Examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## EXAMPLES

### Example 1: Antimicrobial assays

The data for the antimicrobial assay of the peptides have been obtained by making OD measurements in *in vitro* cell culture experiments with and without added peptide. The protocol used is as follows.

Cell lines included *Staphylococcus aureus* ATCC 6538 or 25923, *Pseudomonas aeruginosa* ATCC 9027 or 29853. Medium used were Antibiotic Medium 3 (Difco), Antibiotic Medium 2 (Difco), and 0.85% saline. Controls used were physiological saline, and gentamycin at 50, 25, 10, 5, 1, and 0.1 ppm.

The preparation of all media, stock solutions, and dilutions took place in a laminar flow hood to prevent contamination. Bacterial cells were freshly grown on antibiotic medium 2 agar slants (pH 7.0 at 25 °C). Bacteria were suspended and diluted in antibiotic medium 3 to about $10^4$ cfu/ml and used as the inoculum. Sample solutions (100 µl/well) were added to plates according to the plate layout. Inoculum (100 µl/well) was added to achieve a final concentration of $5 \times 10^3$ cfu/ml. Negative controls received 100 µl saline and 100 µl growth medium. Positive controls received 100 µl saline and 100 µl inoculum. Bacterial plates were incubated at 37 °C for 24 hours.

Absorbance was read at 620 nm after shaking to resuspend cells. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of peptide that completely inhibits the growth of the test organism.

The yeast assay was performed in RPMI 1640 media (pH 7.0 at 25 °C).

The data presented in Table 2 were obtained using the above protocol. However, the data for Table 3 were obtained with a modified protocol wherein the medium was tryptic soy broth, inocolum strength was approximately $10^4$ CFU per ml, and values determined were minimum bactericidal concentrations (MBC) or minimum fungicidal concentrations (MFC).

The following Table 2 describes the antimicrobial properties of the peptides measured as MIC or MFC values in µg/mL. Staph6538 is *Staphylococcus aureus* ATCC accession number 6538; paerug9027 is *Pseudomonas aeruginosa* ATCC accession number 9027, yeast is *Saccharomyces cerevisiae*.

Table 2

| Name | SEQ ID NO: | P Number | staph6538 | paerug9027 | yeast |
|---|---|---|---|---|---|
| Hecate AC #1010 | 1 | 1 | 5 | 10 | > |
| Hecate AM | 2 | 2 | 25 | 100 | 25 |
| SB-37 AC #1018 | 3 | 5 | 100 | 50 | > |
| SB-37 AM | 5 | 12 | > | 100 | > |

| | | | | | |
|---|---|---|---|---|---|
| Shiva 10 AC #1015 | 6 | 13 | 10 | > | > |
| FLAK01 AM | 8 | 23 | 5 | 50 | 100 |
| FLAK04 AM | 10 | 25 | 10 | 5 | 25 |
| FLAK05 AM | 11 | 26 | 10 | 15 | > |
| FLAK06 AM | 12 | 27 | 10 | 10 | 25 |
| KAL V | 15 | 30 | > | > | ND |
| FLAK 17 AM | 16 | 34 | 5 | 50 | 25 |
| FLAK 26 AM | 17 | 35 | 5 | 200 | 25 |
| Hecate 2DAc | 19 | 37 | 5 | 100 | 50 |
| FLAK43 AM | 20 | 38 | 5 | 50 | 50 |
| FLAK44 AM | 21 | 39 | 100 | 25 | 100 |
| FLAK62 AM | 22 | 40 | 100 | 25 | 100 |
| FLAK 06R-AM | 23 | 41 | 10 | 10 | ND |
| MSI-78 AM | 24 | 42 | 10 | > | 200 |
| FLAK50 | 25 | 43 | 5 | 100 | 25 |
| FLAK51 | 26 | 44 | 5 | 5 | 50 |
| FLAK57 | 27 | 45 | 5 | 100 | 100 |
| FLAK71 | 28 | 46 | 10 | 5 | 50 |
| FLAK77 | 29 | 47 | 200 | 100 | 50 |
| FLAK50V | 30 | 48 | 5 | 5 | 25 |
| FLAK50F | 31 | 49 | 10 | 200 | 50 |
| FLAK26V AM | 32 | 50 | 5 | 15 | 50 |
| CAME-15 | 33 | 53 | 5 | 15 | 50 |
| FLAK50C | 34 | 54 | 5 | 50 | 50 |
| FLAK50D | 35 | 55 | 5 | 5 | 25 |
| FLAK 50E | 36 | 56 | 200 | 5 | 50 |
| FLAK80 | 37 | 57 | 100 | 200 | 200 |
| FLAK81 | 38 | 58 | 100 | 100 | 200 |
| FLAK82 | 39 | 59 | > | > | > |
| FLAK83M | 40 | 60 | 200 | 100 | 200 |
| FLAK 17 C | 43 | 64 | 5 | > | 200 |
| FLAK 50H | 44 | 65 | 15 | 50 | 200 |
| FLAK 50G | 45 | 66 | 5 | 50 | 100 |
| Shiva deriv P69+KWKL | 46 | 70 | 10 | > | 100 |
| Shiva 10 (1-18_ AC | 47 | 71 | 15 | 15 | 200 |
| CA(1-7)Shiva10(1-16) | 49 | 73 | 50 | 15 | 100 |
| FLAK 54 | 50 | 74 | 15 | 5 | 100 |
| FLAK 56 | 51 | 75 | 5 | 5 | 50 |
| FLAK 58 | 52 | 76 | 10 | 100 | 200 |
| FLAK 72 | 53 | 77 | 200 | 100 | 200 |

| | | | | | |
|---|---|---|---|---|---|
| FLAK 75 | 54 | 79 | 100 | 200 | 100 |
| Shiva 10 (1-16) Ac | 55 | 80 | 10 | 100 | 100 |
| CA(1-7)Shiva10(1-16)-COOH | 56 | 81 | 10 | > | > |
| Indolocidin-ac | 57 | 91 | 10 | > | > |
| FLAK50B | 58 | 92 | 5 | 5 | 50 |
| FLAK50I | 60 | 94 | 10 | > | > |
| FLAK50K | 61 | 95 | 100 | 200 | > |
| FLAK50L | 62 | 96 | > | > | > |
| Shiva-11 | 63 | 98 | > | > | > |
| Shiva 11[(1-16)ME(2-9)]-COOH | 64 | 99 | 100 | > | > |
| FLAK 50N | 65 | 101 | 10 | 25 | 100 |
| FLAK 50O | 66 | 102 | 5 | 10 | 50 |
| FLAK 50P | 67 | 103 | 10 | 25 | 100 |
| CA(1-&Hecate(11/23) | 68 | 104 | 10 | 10 | 200 |
| PYL-ME | 69 | 105 | 200 | 200 | > |
| FLAG26-D1 | 70 | 106 | 100 | 25 | 100 |
| Vishnu3 | 71 | 107 | > | > | > |
| Melittin | 72 | 108 | 5 | > | 25 |
| FLAK26-D2 | 73 | 109 | > | 200 | 200 |
| FLAG26-D3 | 74 | 110 | > | 200 | 200 |
| FLAK50 Q1 | 75 | 111 | 5 | 100 | 200 |
| FLAK50 Q2 | 76 | 112 | 50 | 200 | 100 |
| FLAK50 Q3 | 77 | 113 | 10 | 200 | 200 |
| FLAK50 Q4 | 78 | 114 | 50 | 15 | 100 |
| FLAK50 Q5 | 79 | 117 | 100 | 200 | 200 |
| FLAK50 Q6 | 80 | 118 | 10 | 100 | 100 |
| FLAK50 Q7 | 81 | 119 | 50 | 25 | 50 |
| FLAK50 Q8 | 82 | 120 | 50 | 200 | 200 |
| FLAK50 Q9 | 83 | 121 | 50 | > | 100 |
| FLAK50 T1 | 85 | 123 | 50 | 200 | 100 |
| FLAK50 T2 | 86 | 124 | 5 | 100 | 100 |
| FLAK50 T3 | 87 | 125 | 10 | 100 | 50 |
| FLAK50 T4 | 88 | 126 | > | > | > |
| FLAK50 T5 | 89 | 127 | 100 | 25 | 100 |
| FLAK90 | 90 | 128 | > | 100 | 200 |
| FLAK91 | 91 | 129 | 100 | 25 | 100 |
| FLAK92 | 92 | 130 | 200 | 200 | 200 |
| FLAK93 | 93 | 131 | 25 | 10 | 100 |
| FLAK50 Z1 | 94 | 132 | > | 100 | > |
| FLAK50 Z2 | 95 | 133 | > | > | > |

| | | | | | |
|---|---|---|---|---|---|
| FLAK50 Z3 | 96 | 134 | 100 | > | 200 |
| FLAK50 Z4 | 97 | 135 | 15 | 10 | 50 |
| FLAK50 Z5 | 98 | 136 | 100 | 50 | 100 |
| FLAK50 Z6 | 99 | 137 | > | > | > |
| FLAK50 Z7 | 100 | 138 | > | > | > |
| FLAK50 Z8 | 101 | 139 | 50 | 25 | 200 |
| FLAK50 Z9 | 102 | 140 | > | > | > |
| FLAK94 | 103 | 141 | 15 | 50 | 200 |
| FLAK93B | 104 | 142 | 100 | 50 | 100 |
| FLAK50 Z10 | 105 | 143 | 100 | 50 | 200 |
| FLAK96 | 106 | 144 | 5 | 50 | 50 |
| FLAK97 | 107 | 145 | 200 | 100 | 200 |
| FLAK98 | 108 | 146 | 10 | 10 | 50 |
| FKRLA | 109 | 147 | 5 | 5 | 200 |
| FLAK91B | 110 | 148 | > | 200 | 200 |
| FLAK92B | 111 | 149 | 50 | 100 | 200 |
| FLAK99 | 112 | 150 | 100 | 10 | > |
| FLAK50T6 | 113 | 151 | > | > | 200 |
| FLAK50T7 | 114 | 152 | 100 | 50 | 100 |
| FLAK95 | 115 | 153 | 5 | 25 | 100 |
| FLAK50T8 | 116 | 154 | 100 | 100 | 50 |
| FLAK50T9 | 117 | 155 | > | > | > |
| FLAK100-CO2H | 118 | 156 | 15 | > | > |
| FAGVL | 119 | 157 | 200 | > | > |
| FLAK120 | 126 | 165 | 10 | 25 | 25 |
| FLAK121 | 127 | 166 | > | > | > |
| FLAK96B | 128 | 167 | 10 | 25 | 100 |
| FLAK96G | 129 | 168 | 50 | 100 | > |
| FLAK96F | 130 | 169 | 100 | 100 | 100 |
| FLAK96C | 131 | 170 | 200 | 100 | 100 |
| FLAK96D | 132 | 171 | 25 | 50 | 100 |
| FLAK 96 | 137 | 176 | > | > | > |
| FLAK 96J | 139 | 178 | 200 | 100 | > |
| FLAK 96L | 140 | 179 | 50 | 50 | 100 |
| FLAK-120G | 141 | 180 | 200 | > | > |
| FLAK-120D | 142 | 181 | 100 | 200 | 100 |
| FLAK-120C | 143 | 182 | > | > | > |
| FLAK-120B | 144 | 183 | 200 | 100 | 200 |
| FLAK-120F | 145 | 184 | 25 | 100 | 100 |
| FLAK 50M | 165 | 97 | 5 | 50 | 50 |

> indicates greater than 200 μg/mL; ND = not determined.

The following Table 3 describes describes the antimicrobial properties of the peptides measured as minimum bactericidal or minimum fungicidal (*Candida*)

concentrations. MBC or MFC values are in μg/mL. E. coli is *Escherichia coli* ATCC accession number 25922; P. aerug is *Pseudomonas aeruginosa* ATCC accession number 27853, S. aur. is *Stapholococcus aureus* ATCC accession number 25923; Candida is *Candida albicans* ATCC accession number 10231.

Table 3

| SEQ ID NO: | P # | E. coli A.25922 | P.aerug A.27853 | S.aur A.25923 | Candida A.10231 |
|---|---|---|---|---|---|
| 1 | 1 | 25 | 30 | 25 | >50 |
| 2 | 2 | 25 | 10 | 25 | >50 |
| 3 | 5 | 50 | >60 | 40 | ND |
| 4 | 11 | 40 | 25 | 25 | >50 |
| 5 | 12 | 50 | >60 | 75 | ND |
| 6 | 13 | 8 | 15 | 30 | >50 |
| 8 | 23 | 15 | 25 | 30 | >50 |
| 9 | 24 | >80 | 30 | >40 | >50 |
| 10 | 25 | 40 | 30 | 40 | >50 |
| 11 | 26 | >80 | >40 | >40 | >50 |
| 12 | 27 | 10 | 8 | 8 | >50 |
| 13 | 27B | 40 | 10 | >40 | >40 |
| 14 | 27C | 10 | 4 | >40 | >40 |
| 15 | 30 | 10 | 15 | 40 | >50 |
| 16 | 34 | 15 | 15 | 40 | >40 |
| 17 | 35 | 8 | 8 | 10 | >40 |
| 18 | 36 | 30 | 15 | 10 | >40 |
| 19 | 37 | 8 | 8 | 40 | >50 |
| 20 | 38 | 15 | 30 | 15 | ND |
| 21 | 39 | >40 | >40 | >40 | ND |
| 22 | 40 | 30 | 40 | >40 | ND |
| 23 | 41 | 40 | 40 | 40 | ND |
| 24 | 42 | 10 | 30 | 10 | ND |
| 25 | 43 | 8 | 15 | 4 | 15 |
| 26 | 44 | 10 | 55 | 30 | >50 |
| 27 | 45 | 30 | 40 | 80 | >50 |
| 29 | 47 | >50 | >50 | >50 | >50 |
| 30 | 48 | 8 | 25 | 4 | 10 |
| 31 | 49 | 40 | 30 | 50 | 30 |
| 32 | 50 | 50 | 25 | 25 | >50 |
| 33 | 53 | 15 | 15 | 10 | 30 |
| 34 | 54 | 15 | 40 | 15 | 30 |
| 35 | 55 | 4 | 10 | 4 | 25 |
| 36 | 56 | 50 | 10 | 55 | 30 |
| 37 | 57 | >50 | >50 | >50 | >50 |

| | | | | | |
|---|---|---|---|---|---|
| 38 | 58 | >50 | >50 | >50 | >50 |
| 39 | 59 | >50 | >50 | >50 | >50 |
| 40 | 60 | >50 | >50 | >50 | >50 |
| 41 | 61 | 4 | 50 | >80 | >40 |
| 42 | 63 | 10 | 50 | 15 | 60 |
| 43 | 64 | 10 | 30 | 4 | >50 |
| 44 | 65 | >55 | >50 | >55 | >50 |
| 45 | 66 | 40 | 50 | 30 | 40 |
| 46 | 70 | 40 | 30 | 40 | >50 |
| 47 | 71 | 50 | 40 | >50 | >50 |
| 48 | 72 | >50 | 40 | >50 | >50 |
| 50 | 74 | >55 | 50 | >55 | >55 |
| 51 | 75 | 40 | 30 | >55 | 30 |
| 52 | 76 | 40 | >55 | >55 | >50 |
| 53 | 77 | >50 | >50 | >50 | >50 |
| 54 | 79 | >50 | >50 | >50 | >50 |
| 55 | 80 | 30 | 15 | >50 | >50 |
| 58 | 92 | 40 | 25 | 15 | 25 |
| 59 | 93 | >50 | >50 | >50 | >50 |
| 60 | 94 | >50 | >50 | >50 | >50 |
| 61 | 95 | >50 | >50 | >50 | >50 |
| 62 | 96 | >50 | >50 | >50 | >50 |
| 65 | 101 | 300 | >50 | >50 | 40 |
| 66 | 102 | 25 | 30 | 25 | 15 |
| 67 | 103 | 30 | 30 | >50 | 25 |
| 69 | 105 | 25 | >50 | ND | >50 |
| 70 | 106 | 50 | >50 | ND | >50 |
| 71 | 107 | ND | >50 | >50 | >50 |
| 72 | 108 | >50 | >50 | 25 | >50 |
| 73 | 109 | ND | ND | 80 | >50 |
| 74 | 110 | 8 | >50 | >50 | >50 |
| 75 | 111 | 30 | ND | 40 | INACT |
| 76 | 112 | 30 | INACT | INACT | INACT |
| 77 | 113 | INACT | INACT | INACT | 40 |
| 79 | 117 | INACT | INACT | INACT | INACT |
| 80 | 118 | 8 | 25 | 10 | 25 |
| 81 | 119 | 15 | 30 | 4 | 25 |
| 82 | 120 | INACT | INACT | INACT | INACT |
| 83 | 121 | INACT | INACT | INACT | 50 |
| 84 | 122 | 30 | 30 | 25 | 15 |
| 85 | 123 | 40 | INACT | INACT | 25 |
| 86 | 124 | 10 | 40 | 8 | 15 |
| 87 | 125 | 40 | 40 | INACT | 40 |
| 88 | 126 | INACT | INACT | INACT | INACT |

| | | | | | |
|---|---|---|---|---|---|
| 89 | 127 | INACT | INACT | INACT | INACT |
| 90 | 128 | INACT | INACT | INACT | INACT |
| 91 | 129 | INACT | INACT | INACT | INACT |
| 92 | 130 | INACT | INACT | INACT | INACT |
| 93 | 131 | INACT | INACT | INACT | INACT |
| 94 | 132 | INACT | INACT | INACT | INACT |
| 95 | 133 | INACT | INACT | INACT | INACT |
| 96 | 134 | INACT | INACT | INACT | 25 |
| 97 | 135 | INACT | 40 | INACT | INACT |
| 98 | 136 | INACT | INACT | INACT | INACT |
| 99 | 137 | INACT | INACT | INACT | INACT |
| 100 | 138 | INACT | INACT | INACT | INACT |
| 101 | 139 | INACT | INACT | INACT | INACT |
| 102 | 140 | INACT | INACT | INACT | INACT |
| 103 | 141 | INACT | INACT | INACT | INACT |
| 104 | 142 | INACT | INACT | INACT | INACT |
| 105 | 143 | INACT | INACT | INACT | INACT |
| 106 | 144 | 10 | 25 | 25 | 25 |
| 107 | 145 | INACT | INACT | INACT | 100 |
| 108 | 146 | 10 | >250 | 75 | 10 |
| 109 | 147 | 25 | 75 | >250 | >250 |
| 110 | 148 | 150 | >250 | >250 | 100 |
| 111 | 149 | 150 | >250 | >250 | 100 |
| 112 | 150 | 75 | >250 | >250 | 50 |
| 113 | 151 | >250 | >250 | >250 | 100 |
| 114 | 152 | 150 | 150 | >250 | 50 |
| 115 | 153 | 10 | 25 | 5 | 25 |
| 116 | 154 | 50 | 100 | >250 | 25 |
| 117 | 155 | >250 | >250 | >250 | >250 |
| 118 | 156 | 100 | >250 | >250 | >250 |
| 119 | 157 | 75 | >250 | >250 | >250 |
| 120 | 159 | 10 | 10 | >250 | 50 |
| 121 | 160 | >250 | >250 | >250 | >250 |
| 122 | 161 | 150 | >250 | >250 | 25 |
| 123 | 162 | 50 | >250 | >250 | 100 |
| 124 | 163 | 25 | 50 | 25 | 25 |
| 125 | 164 | 25 | 25 | 25 | 25 |
| 126 | 165 | 10 | 25 | 25 | 10 |
| 127 | 166 | >250 | >250 | >250 | >250 |
| 128 | 167 | 25 | >250 | 10 | 25 |
| 129 | 168 | 75 | 100 | >250 | 150 |
| 130 | 169 | 200 | >250 | >250 | 75 |
| 131 | 170 | 25 | >250 | 150 | 25 |
| 132 | 171 | 75 | 100 | >250 | 50 |

| | | | | | |
|---|---|---|---|---|---|
| 133 | 172 | >250 | >250 | >250 | >250 |
| 134 | 173 | >250 | >250 | >250 | 150 |
| 162 | 67 | 25 | 30 | 30 | >50 |
| 165 | 97 | 25 | >50 | 25 | 25 |

INACT refers to no detectable activity. ND indicates no data available.

Example 2: Anti-cancer assays

Cancer cell assays were performed in a manner similar to the anti-microbial assays described above, except that the assay procedure used the MTT dye protocol. Viability of cells is determined by the dye response. In the following procedure, approximately 1.5 x $10^4$ cells per well were added and viability was determined with the cells in a semi-confluent state. The assay was performed in a 96-well microtiter plate. After addition of peptide, the plate was set for 24 hours. MTT (5 mg/ml in phenol red-free RPMI-1640, 20 µl) was added to each well including positive control wells untreated with peptide. The plate was incubated at 37 °C for 4 hours. The liquid contents of each well was removed, and isopropanol with 0.1 M HCl (100 µl) was added to each well. The plate was sealed with parafilm to prevent evaporation of the isopropanol. The plate is allowed to rest for 5-10 minutes in order to solubilize the precipitate. Purified water (100 µl) was added to each well. Absorbance was determined with an ELISA Reader instrument. Color intensity at 540 nm is proportional to viability of cells. Results for each concentration of peptide are plotted relative to untreated controls, and LD50 values are determined from the graphs.

WI38 (ATCC No. CCL75) is a normal fibroblast line of lung diploid cells, MCF7 (ATCC No. HTB22) is a breast adenocarcinoma tumor cell line, SW480 (ATCC No. CCL228) is a colon adenocarcinoma tumor cell line, BMKC is a cloned melanoma line derived from Bowes melanoma line HMCB (ATCC No. CRL9607), H1299 (ATCC No. CRL5803) is a lung large cell carcinoma tumor line, HeLaS3 (ATCC No. CCL2.2) is a cervical epitheleal carcinoma tumor cell line, and PC3 (ATCC No. CRL1435) is a prostate adenocarcinoma tumor cell line. Numbers are $LD_{50}$ values (µg/mL). Data on the six targets are presented in the following Tables 4 and 5.

Table 4

| Name | SEQ ID NO: | P No. | WI38 | MCF7 | SW480 | BMKC |
|---|---|---|---|---|---|---|
| HECATE AC | 1 | 1 | 27 | 54 | 6 | 72 |
| HECATE AM | 2 | 2 | 66 | 23 | 46 | 128 |
| SB37COOH | 3 | 5 | 130 | 175 | 82 | 120 |
| SB-37 AM | 5 | 12 | 950 | 540 | > | > |
| SHIVA 10 AC | 6 | 13 | 57 | > | ND | ND |
| FLAK01 AM | 8 | 23 | 34 | 62 | 5 | 27 |
| FLAK03 AM | 9 | 24 | 55 | 26 | 38 | 85 |
| FLAK04 AM | 10 | 25 | 24 | 10 | 12 | 36 |
| FLAK05 AM | 11 | 26 | 96 | 74 | 8 | 94 |
| FLAK06 AM | 12 | 27 | 37 | 14 | 26 | 44 |
| FLAK06 AC | 13 | 27B | 101 | 65 | 59 | 93 |
| FLAK06 R-AC | 14 | 27C | 520 | 140 | 210 | 300 |
| KAL V | 15 | 30 | 93 | 72 | 62 | 140 |
| FLAK 17 AM | 16 | 34 | 40 | 21 | 35 | 53 |
| FLAK 26 AM | 17 | 35 | 8 | 9 | 14 | 7 |
| FLAK 25 AM | 18 | 36 | 19 | 9 | 30 | 56 |
| HECATE 2DAc | 19 | 37 | 80 | 14 | 57 | 150 |
| FLAK43 AM | 20 | 38 | 12 | 17 | 13 | 21 |
| FLAK44 AM | 21 | 39 | 300 | 130 | 435 | 510 |
| FLAK62 AM | 22 | 40 | > | 760 | > | > |
| FLAK 06R-AM | 23 | 41 | 175 | 98 | 120 | 290 |
| MSI-78 AM | 24 | 42 | 67 | 31 | 34 | 140 |
| FLAK50 | 25 | 43 | 5 | 9 | 9 | 7 |
| FLAK51 | 26 | 44 | 36 | 140 | 32 | 47 |
| FLAK57 | 27 | 45 | 200 | 260 | 180 | 160 |
| FLAK71 | 28 | 46 | 200 | 300 | 160 | 150 |
| FLAK77 | 29 | 47 | > | 575 | > | 700 |
| FLAK50V | 30 | 48 | 41 | 23 | 47 | 43 |
| FLAK50F | 31 | 49 | 135 | 40 | 100 | 115 |
| FLAK26V AM | 32 | 50 | 43 | 32 | 46 | 40 |
| CAME-15 | 33 | 53 | 32 | 45 | | 40 |
| FLAK50C | 34 | 54 | 97 | 60 | | 90 |
| FLAK50D | 35 | 55 | 32 | 16 | 14 | 16 |
| FLAK 50E | 36 | 56 | 250 | 500 | 215 | 205 |
| FLAK80 | 37 | 57 | 900 | > | 740 | 740 |
| FLAK81 | 38 | 58 | > | > | > | > |
| FLAK82 | 39 | 59 | 77 | 31 | 42 | 155 |
| FLAK83M | 40 | 60 | > | > | > | > |
| FLAK 26 Ac | 41 | 61 | 93 | 105 | 100 | 140 |
| INDOLICIDIN | 42 | 63 | ND | 64 | 345 | 200 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | 80 | | 35 |
| FLAK 17 C | 43 | 64 | 37 | 475 | 345 | 250 |
| FLAK 50H | 44 | 65 | 320 | 90 | 145 | 200 |
| FLAK 50G | 45 | 66 | 240 | 44 | 11 | 94 |
| SHIVA DERIV P69+KWKL | 46 | 70 | 34 | 190 | 250 | 445 |
| SHIVA 10 (1-18_ AC | 47 | 71 | 355 | 93 | 82 | 290 |
| SHIVA 10 PEPTIDE 71+KWKL | 48 | 72 | 125 | 150 | 70 | 360 |
| CA(1-7)Shiva10(1-16) | 49 | 73 | 160 | 465 | 340 | 460 |
| FLAK 54 | 50 | 74 | 335 | 42 | 17 | 24 |
| FLAK 56 | 51 | 75 | 80 | 970 | 400 | 750 |
| FLAK 58 | 52 | 76 | 445 | > | > | 125 |
| FLAK 72 | 53 | 77 | > | 540 | > | 830 |
| FLAK 75 | 54 | 79 | > | 29 | 35 | 76 |
| SHIVA 10 (1-16) Ac | 55 | 80 | 28 | 63 | 13 | 12 |
| CA(1-7)Shiva10(1-16)-COOH | 56 | 81 | 8 | 12 | 30 | 180 |
| INDOLOCIDIN-ac | 57 | 91 | 9 | 23 | 51 | 46 |
| FLAK50B | 58 | 92 | 43 | 65 | ND | 11 |
| FLAK50I | 60 | 94 | 6 | > | > | 820 |
| FLAK50K | 61 | 95 | 250 | > | > | > |
| FLAK50L | 62 | 96 | > | 96 | 125 | 94 |
| Shiva-11 | 63 | 98 | 47 | 95 | 120 | 94 |
| SHIVA 11 [(1-16)ME(2-9] -COOH | 64 | 99 | 34 | 250 | 170 | 160 |
| FLAK 50N | 65 | 101 | 300 | 60 | 57 | 60 |
| FLAK 50O | 66 | 102 | 73 | 46 | 90 | 75 |
| FLAK 50P | 67 | 103 | 26 | 11 | 54 | 100 |
| CA(1-&HECATE(11/23) | 68 | 104 | 24 | 635 | > | ND |
| PYL-ME | 69 | 105 | 430 | 620 | 570 | 690 |
| FLAG26-D1 | 70 | 106 | > | > | > | > |
| VISHNU3 | 71 | 107 | > | 9 | 23 | 18 |
| MELITTIIN | 72 | 108 | 16 | > | > | > |
| FLAK26-D2 | 73 | 109 | > | 180 | 325 | 400 |
| FLAG26-D3 | 74 | 110 | 45 | 35 | 27 | 26 |
| FLAK50 Q1 | 75 | 111 | 24 | 500 | 800 | 445 |
| FLAK50 Q2 | 76 | 112 | 420 | 150 | 180 | 115 |
| FLAK50 Q3 | 77 | 113 | 170 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| FLAK50 Q4 | 78 | 114 | > | 730 | > | > |
| FLAK50 Q5 | 79 | 117 | > | > | > | > |
| FLAK50 Q6 | 80 | 118 | 170 | 70 | 115 | 135 |
| FLAK50 Q7 | 81 | 119 | 45 | 54 | 46 | 36 |
| FLAK50 Q8 | 82 | 120 | 600 | 730 | 630 | 660 |
| FLAK50 Q9 | 83 | 121 | 625 | 400 | 800 | 670 |
| FLAK50 Q10 | 84 | 122 | 720 | 360 | 570 | 700 |
| FLAK50 T1 | 85 | 123 | 600 | 615 | > | 635 |
| FLAK50 T2 | 86 | 124 | 21 | 18 | 9 | 10 |
| FLAK50 T3 | 87 | 125 | 90 | 90 | 125 | 220 |
| FLAK50 T4 | 88 | 126 | > | > | > | > |
| FLAK50 T5 | 89 | 127 | 760 | 440 | 400 | 535 |
| FLAK90 | 90 | 128 | 500 | 500 | 530 | 330 |
| FLAK91 | 91 | 129 | > | > | 550 | > |
| FLAK92 | 92 | 130 | > | > | > | > |
| FLAK93 | 93 | 131 | > | 600 | 555 | > |
| FLAK50 Z1 | 94 | 132 | > | > | > | > |
| FLAK50 Z2 | 95 | 133 | > | > | > | > |
| FLAK50 Z3 | 96 | 134 | > | > | 740 | > |
| FLAK50 Z4 | 97 | 135 | 110 | 54 | 80 | 155 |
| FLAK50 Z5 | 98 | 136 | > | 500 | 600 | 530 |
| FLAK50 Z6 | 99 | 137 | > | > | > | > |
| FLAK50 Z7 | 100 | 138 | > | > | > | > |
| FLAK50 Z8 | 101 | 139 | 550 | 625 | > | 525 |
| FLAK50 Z9 | 102 | 140 | > | > | > | > |
| FLAK94 | 103 | 141 | 420 | 430 | 560 | 465 |
| FLAK93B | 104 | 142 | 73 | 44 | 38 | 38 |
| FLAK50 Z10 | 105 | 143 | > | > | > | > |
| FLAK96 | 106 | 144 | 750 | 150 | 285 | 250 |
| FLAK97 | 107 | 145 | > | > | > | > |
| FLAK98 | 108 | 146 | 270 | 110 | 380 | 185 |
| FKRLA | 109 | 147 | 83 | 106 | 185 | 110 |
| FLAK91B | 110 | 148 | 380 | 315 | > | 330 |
| FLAK92B | 111 | 149 | > | > | > | > |
| FLAK99 | 112 | 150 | 125 | 160 | 235 | 190 |
| FLAK50T6 | 113 | 151 | > | > | > | > |
| FLAK50T7 | 114 | 152 | 620 | 430 | 740 | > |
| FLAK95 | 115 | 153 | 130 | 64 | 61 | 165 |
| FLAK50T8 | 116 | 154 | 600 | 315 | 750 | 330 |
| FLAK50T9 | 117 | 155 | > | > | > | > |
| FLAK100-CO2H | 118 | 156 | 230 | 135 | 345 | 520 |
| FAGVL | 119 | 157 | 500 | 240 | 530 | 600 |
| Modelin-5 | 120 | 159 | 82 | 61 | 140 | 140 |
| Modelin-5-CO2H | 121 | 160 | 700 | 320 | 370 | 220 |

| | | | | | | |
|---|---|---|---|---|---|---|
| FLAK120 | 126 | 165 | 470 | 360 | 240 | 240 |
| FLAK121 | 127 | 166 | > | > | > | > |
| FLAK96B | 128 | 167 | 260 | 230 | 360 | 240 |
| FLAK96G | 129 | 168 | > | 630 | > | 590 |
| FLAK96F | 130 | 169 | > | 510 | > | 530 |
| FLAK96C | 131 | 170 | > | 940 | > | > |
| FLAK96D | 132 | 171 | 615 | 305 | 770 | 600 |
| Modelin-8D | 135 | 174 | > | > | > | > |
| Modelin-8E | 136 | 175 | > | > | 70 | > |
| Flak 96H | 137 | 176 | > | > | > | > |
| Flak 96I | 138 | 177 | 270 | 190 | 310 | 310 |
| Flak 96J | 139 | 178 | 405 | 770 | > | 640 |
| Flak 96L | 140 | 179 | 540 | 555 | > | 920 |
| FLAK-120G | 141 | 180 | 940 | 950 | 600 | 770 |
| FLAK-120D | 142 | 181 | 500 | 550 | 870 | 830 |
| FLAK-120C | 143 | 182 | > | > | > | > |
| FLAK-120B | 144 | 183 | > | > | > | > |
| FLAK-120F | 145 | 184 | 800 | 260 | 440 | 600 |
| Magainin2wisc | 146 | 300 | 52 | 22 | 60 | 130 |
| D2A21 | 147 | 301 | 66 | 64 | 76 | 140 |
| KSL-1 | 148 | 302 | 800 | 340 | > | 700 |
| KSL-7 | 149 | 303 | 355 | 315 | 530 | 330 |
| LSB-37 | 150 | 306 | 320 | 50 | 240 | 170 |
| Anubis-2 | 151 | 307 | 75 | 38 | 73 | 83 |
| FLAK 17 CV | 152 | 501 | 26 | 23 | ND | ND |
| FLAK50 Q1V | 153 | 502 | 64 | 92 | ND | ND |
| D2A21V | 154 | 503 | 150 | 210 | ND | ND |
| FLAK 25 AM V | 155 | 504 | 110 | 130 | ND | ND |
| FLAK43 AM V | 156 | 505 | 85 | 86 | ND | ND |
| FLAK50D V | 157 | 506 | 75 | 45 | ND | ND |
| HECATE AM V | 158 | 507 | 285 | 340 | ND | ND |
| HECATE AC V | 159 | 508 | 190 | 160 | ND | ND |
| FLAK04 AM V | 160 | 509 | 95 | 84 | ND | ND |
| 03 AM V | 161 | 510 | 77 | 62 | ND | ND |
| | 162 | 67 | 4 | 7 | ND | ND |
| | 163 | 100 | 95 | 175 | 82 | 120 |
| | 164 | 69 | 101 | 45 | 63 | 66 |

Note: > indicates greater than 1000; ND indicates not determined; numbers are in μg/mL.

Table 5

| Name | SEQ ID NO: | P No. | WI38 | H1299 | HeLaS3 | PC3 |
|---|---|---|---|---|---|---|
| HECATE AC | 1 | 1 | 27 | 44 | 95 | 61 |
| HECATE AM | 2 | 2 | 66 | 140 | 50 | 44 |
| SB37COOH | 3 | 5 | 130 | 220 | 150 | ND |
| SB-37 AM | 5 | 12 | 950 | 720 | > | 630 |
| SHIVA 10 AC | 6 | 13 | 57 | > | > | 83 |
| FLAK01 AM | 8 | 23 | 34 | 64 | 82 | 41 |
| FLAK03 AM | 9 | 24 | 55 | 72 | 145 | 38 |
| FLAK04 AM | 10 | 25 | 24 | 37 | 20 | 12 |
| FLAK05 AM | 11 | 26 | 96 | 84 | 150 | 125 |
| FLAK06 AM | 12 | 27 | 37 | 16 | 25 | 8 |
| FLAK06 AC | 13 | 27B | 101 | 54 | 80 | 16 |
| FLAK06 AM | 14 | 27C | 520 | 170 | 260 | 280 |
| KAL V | 15 | 30 | 93 | 125 | 190 | 65 |
| FLAK 17 AM | 16 | 34 | 40 | 24 | 62 | 9 |
| FLAK 26 AM | 17 | 35 | 8 | 16 | 27 | 5 |
| FLAK 25 AM | 18 | 36 | 19 | 57 | ND | 19 |
| HECATE 2DAc | 19 | 37 | 80 | 150 | ND | 64 |
| FLAK43 AM | 20 | 38 | 12 | 33 | 35 | 10 |
| FLAK44 AM | 21 | 39 | 300 | 420 | 620 | 310 |
| FLAK62 AM | 22 | 40 | > | > | > | 435 |
| FLAK 06R-AM | 23 | 41 | 175 | 245 | 185 | 140 |
| MSI-78 AM | 24 | 42 | 67 | 150 | ND | 66 |
| FLAK50 | 25 | 43 | 5 | 6 | 15 | 12 |
| FLAK51 | 26 | 44 | 36 | 72 | 22 | 45 |
| FLAK57 | 27 | 45 | 200 | 330 | 160 | 170 |
| FLAK71 | 28 | 46 | 200 | 290 | 280 | 280 |
| FLAK77 | 29 | 47 | > | > | > | > |
| FLAK50V | 30 | 48 | 41 | 17 | 44 | 32 |
| FLAK50F | 31 | 49 | 135 | 140 | ND | 77 |
| FLAK26V AM | 32 | 50 | 43 | 7 | 33 | 54 |
| CAME-15 | 33 | 53 | 32 | 65 | 30 | 40 |
| FLAK50C | 34 | 54 | 97 | 80 | 190 | 90 |
| FLAK50D | 35 | 55 | 32 | 7 | 15 | 47 |
| FLAK 50E | 36 | 56 | 250 | 370 | 300 | 435 |
| FLAK80 | 37 | 57 | 900 | > | 830 | > |
| FLAK81 | 38 | 58 | > | > | > | > |
| FLAK82 | 39 | 59 | 77 | 180 | ND | 81 |
| FLAK83M | 40 | 60 | > | > | > | > |
| FLAK 26 Ac | 41 | 61 | 93 | 127 | 170 | 66 |
| INDOLICIDIN | 42 | 63 | ND | 270 | 345 | 290 |
| FLAK 17 C | 43 | 64 | 37 | 30 | 30 | 46 |

| | | | | | | |
|---|---|---|---|---|---|---|
| FLAK 50H | 44 | 65 | 320 | 450 | 210 | 470 |
| FLAK 50G | 45 | 66 | 240 | 130 | 140 | 170 |
| SHIVA DERIV P69+KWKL | 46 | 70 | 34 | 63 | 28 | 82 |
| SHIVA 10 (1-18_ AC | 47 | 71 | 355 | 320 | 570 | 270 |
| SHIVA 10 PEPTIDE 71+KWKL | 48 | 72 | 125 | 160 | 240 | 63 |
| CA(1-7)Shiva10(1-16) | 49 | 73 | 160 | 115 | 270 | 97 |
| FLAK 54 | 50 | 74 | 335 | 670 | 260 | 660 |
| FLAK 56 | 51 | 75 | 80 | 80 | 74 | 54 |
| FLAK 58 | 52 | 76 | 445 | 860 | 380 | 675 |
| FLAK 72 | 53 | 77 | > | > | > | > |
| FLAK 75 | 54 | 79 | > | > | > | > |
| SHIVA 10 (1-16) Ac | 55 | 80 | 28 | 64 | 97 | 28 |
| CA(1-7)Shiva10(1-16)-COOH | 56 | 81 | 8 | 22 | 19 | 170 |
| Indolocidin-ac | 57 | 91 | 9 | 64 | 20 | 31 |
| FLAK50B | 58 | 92 | 43 | 25 | 670 | 83 |
| FLAK50J | 59 | 93 | 530 | 320 | > | 690 |
| FLAK50I | 60 | 94 | 6 | ND | > | ND |
| FLAK50K | 61 | 95 | 250 | > | > | > |
| FLAK50L | 62 | 96 | > | > | > | > |
| Shiva-11 | 63 | 98 | 47 | 53 | 175 | 52 |
| SHIVA 11 [(1-16)ME(2-9] -COOH | 64 | 99 | 34 | 54 | 180 | 28 |
| FLAK 50N | 65 | 101 | 300 | 340 | 170 | 730 |
| FLAK 50O | 66 | 102 | 73 | 27 | 43 | 66 |
| FLAK 50P | 67 | 103 | 26 | 150 | 70 | 330 |
| CA(1-&HECATE(11/23) | 68 | 104 | 24 | 52 | 130 | 18 |
| PYL-ME | 69 | 105 | 430 | > | > | ND |
| FLAG26-D1 | 70 | 106 | > | 920 | 700 | > |
| VISHNU3 | 71 | 107 | > | > | > | > |
| MELITTIIN | 72 | 108 | 16 | 25 | 35 | 13 |
| FLAK26-D2 | 73 | 109 | > | > | > | > |
| FLAG26-D3 | 74 | 110 | 45 | 95 | 540 | > |
| FLAK50 Q1 | 75 | 111 | 24 | 8 | 7 | 11 |
| FLAK50 Q2 | 76 | 112 | 420 | 470 | 660 | 640 |
| FLAK50 Q3 | 77 | 113 | 170 | 50 | 190 | 240 |

| | | | | | | |
|---|---|---|---|---|---|---|
| FLAK50 Q4 | 78 | 114 | > | > | > | > |
| FLAK50 Q5 | 79 | 117 | > | > | > | > |
| FLAK50 Q6 | 80 | 118 | 170 | 74 | 87 | 330 |
| FLAK50 Q7 | 81 | 119 | 45 | 33 | 30 | 140 |
| FLAK50 Q8 | 82 | 120 | 600 | 620 | 810 | > |
| FLAK50 Q9 | 83 | 121 | 625 | 460 | 830 | > |
| FLAK50 Q10 | 84 | 122 | 720 | 830 | 780 | 800 |
| FLAK50 T1 | 85 | 123 | 600 | > | 940 | > |
| FLAK50 T2 | 86 | 124 | 21 | 30 | 14 | 10 |
| FLAK50 T3 | 87 | 125 | 90 | 76 | 220 | 145 |
| FLAK50 T4 | 88 | 126 | > | > | > | > |
| FLAK50 T5 | 89 | 127 | 760 | 770 | 610 | > |
| FLAK90 | 90 | 128 | 500 | > | 700 | > |
| FLAK91 | 91 | 129 | > | 790 | 550 | > |
| FLAK92 | 92 | 130 | > | > | > | > |
| FLAK93 | 93 | 131 | > | > | > | > |
| FLAK50 Z1 | 94 | 132 | > | > | > | > |
| FLAK50 Z2 | 95 | 133 | > | > | > | > |
| FLAK50 Z3 | 96 | 134 | > | > | > | > |
| FLAK50 Z4 | 97 | 135 | 110 | 115 | 215 | 310 |
| FLAK50 Z5 | 98 | 136 | > | 450 | 400 | 900 |
| FLAK50 Z6 | 99 | 137 | > | > | > | > |
| FLAK50 Z7 | 100 | 138 | > | > | > | > |
| FLAK50 Z8 | 101 | 139 | 550 | 850 | > | > |
| FLAK50 Z9 | 102 | 140 | > | > | 285 | > |
| FLAK94 | 103 | 141 | 420 | > | > | ND |
| FLAK93B | 104 | 142 | 73 | 115 | 55 | 60 |
| FLAK50 Z10 | 105 | 143 | > | > | > | > |
| FLAK96 | 106 | 144 | 750 | 225 | 275 | 350 |
| FLAK97 | 107 | 145 | > | > | 240 | > |
| FLAK98 | 108 | 146 | 270 | 93 | 640 | 440 |
| FKRLA | 109 | 147 | 83 | 93 | > | 340 |
| FLAK91B | 110 | 148 | 380 | 660 | > | > |
| FLAK92B | 111 | 149 | > | > | > | > |
| FLAK99 | 112 | 150 | 125 | 185 | 320 | 74 |
| FLAK50T6 | 113 | 151 | > | > | > | > |
| FLAK50T7 | 114 | 152 | 620 | 410 | > | > |
| FLAK95 | 115 | 153 | 130 | 50 | 140 | 97 |
| FLAK50T8 | 116 | 154 | 600 | 400 | > | 640 |
| FLAK50T9 | 117 | 155 | > | > | > | ND |
| FLAK100-CO2H | 118 | 156 | 230 | ND | > | 260 |
| FAGVL | 119 | 157 | 500 | 315 | > | 375 |
| Modelin-5 | 120 | 159 | 82 | 74 | 275 | 145 |
| Modelin-5-CO2H | 121 | 160 | 700 | 470 | 550 | 450 |

| | | | | | | |
|---|---|---|---|---|---|---|
| FLAK120 | 126 | 165 | 470 | 56 | 400 | 340 |
| FLAK121 | 127 | 166 | > | > | > | > |
| FLAK96B | 128 | 167 | 260 | 300 | 325 | 320 |
| FLAK96G | 129 | 168 | > | > | > | > |
| FLAK96F | 130 | 169 | > | 640 | > | > |
| FLAK96C | 131 | 170 | > | > | > | > |
| FLAK96D | 132 | 171 | 615 | 540 | 820 | 600 |
| Modelin-8D | 135 | 174 | > | > | > | > |
| Modelin-8E | 136 | 175 | > | > | 510 | > |
| Flak 96H | 137 | 176 | > | > | > | > |
| Flak 96I | 138 | 177 | 270 | 240 | 380 | 120 |
| Flak 96J | 139 | 178 | 405 | > | > | > |
| Flak 96L | 140 | 179 | 540 | > | > | > |
| FLAK-120G | 141 | 180 | 940 | > | 760 | > |
| FLAK-120D | 142 | 181 | 500 | > | > | > |
| FLAK-120C | 143 | 182 | > | > | > | > |
| FLAK-120B | 144 | 183 | > | > | > | > |
| FLAK-120F | 145 | 184 | 800 | 370 | 302 | 570 |
| Magainin2wisc | 146 | 300 | 52 | 60 | 125 | 45 |
| D2A21 | 147 | 301 | 66 | 77 | 170 | 45 |
| KSL-1 | 148 | 302 | 800 | 720 | > | > |
| KSL-7 | 149 | 303 | 355 | 345 | > | 530 |
| LSB-37 | 150 | 306 | 320 | 120 | 250 | 370 |
| Anubis-2 | 151 | 307 | 75 | 160 | 100 | 66 |
| | 163 | 100 | 95 | 220 | 150 | ND |
| | 164 | 69 | 101 | 71 | 190 | 81 |

Note: > indicates greater than 1000; ND indicates not determined; numbers are in μg/mL.

It can be seen from Tables 4 and 5 that all targets challenged were inhibited by one or more of the peptides to an appreciable extent (i.e. LD50 less than 50 μg/ml). Table 6 below shows that 44 (29%) of the 150 peptides tested were active with some LD50 values at or below 50; 26 of the peptides were active on some targets at or below the LD50 value of 25; and 16 peptides were very active on one or more target strains with LD50 values at or below 10.

Table 7 below shows a broad spectrum of activity against six cancer cell types for various active peptides. It is noted that each target has one or more lead candidate peptides inhibitory to cell growth at an LD50 level of 10 or less.

Table 6: FLAK peptides showing substantial activity against cancer cell lines

| LD50 values | Number of "active" peptides | Percent of 150 peptides tested |
|---|---|---|
| < or = 50 μg/ml | 44 | 29% |
| < or = 25 μg/ml | 26 | 17% |
| < or = 10 μg/ml | 16 | 11% |

Table 7: Activity and specificity of FLAK peptides against six cancer cell targets

| LD50 | Number of active peptides per target | | | | | |
|---|---|---|---|---|---|---|
| | MCF7 (breast) | SW480 (colon) | BMKC (melanoma) | H1299 (lung) | HeLaS3 (cervix) | PC3 (prostate) |
| < or = 50 μg/ml | 31 | 25 | 19 | 19 | 17 | 20 |
| < or = 25 μg/ml | 17 | 13 | 8 | 10 | 8 | 11 |
| < or = 10 μg/ml | 6 | 5 | 3 | 4 | 1 | 5 |

Example 3: Stimulation and proliferation of leukocytes

*In vitro* viability of human leukocyte cells in the presence of different peptides at different concentrations was determined by an Alamar Blue protocol. Alamar Blue (Promega, Madison, WI) is an indicator dye, formulated to measure quantitatively the proliferation and cytotoxicity of the cells. The dye consists of an oxidation-reduction (redox) indicator that yields a colorimetric change and a fluorescent signal in response to cellular metabolic activity.

Assay protocol: Blood from a 50 year old male human was drawn and centrifuged at 1500 rpm for 15 minutes at room temperature. The buffy coat cells at the plasma-red blood cell interface were aspirated. Buffy coat cells (mainly lymphocyte cells) were then transferred into 15 ml centrifuge tubes containing 5 ml of RPMI-1640 medium+10% Fetal Bovine Serum (Gibco, Grand Island, NY). Additional medium was added to the tubes to bring the volume up to 10 ml. The buffy coat suspension was then carefully layered on 5 ml of Histopaque (Sigma Chemical Co., St. Louis, MO) and centrifuged at 1500 rpm for 30 minutes at room temperature. The interface which is mostly PBMCs (peripheral mononuclear cells) was aspirated and transferred to a 15 ml conical centrifuge tube and, resuspended in 2 ml cold RPMI-1640 and brought up to 15 ml with cold RPMI-1640 medium. Cells were centrifuged at 1500 rpm for 10 minutes. The supernatant was then aspirated and discarded. The cell pellet was re-suspended in 1 ml of cold RPMI

1640 and brought up to 15 ml with RPMI medium. This step was repeated twice, except that in the last step, the cells were resuspended with 1 ml of cold RPMI-1640 medium and cell counts were performed with a hemocytometer according to the Sigma cell culture catalogue.

Pokewood mitogen was used as a control along with positive and negative controls. Negative control cells were killed with 70% methanol. Positive (+) control cells were incubated in RPMI medium (untreated). 20 ml of AlamarBlue was added to the cells, and readings were taken after 24 hours, 48 hours, 72 hours, and 96 hours using a fluorimeter (excitation 544/transmission 590 nm).

Calculations were performed using the following formula:

$$\% \text{ treated cell viability} = \frac{\text{Peptide treated sample (adj. for negative control)}}{\text{Positive control (adj. for negative control)}} \times 100\%$$

Using the protocol described immediately above, about 100-150 peptides were screened for their stimulatory and/or inhibitory actions upon the growth of human leukocyte ("WBC") cells as compared to the growth of untreated positive control cells. The data in Table 8 below show that various selected FLAK peptides are stimulatory at low concentrations (0.1 to 1.0 μg/ml), whereas certain of the peptides become inhibitory (causing cell death) at higher concentrations. Several of the peptides (i.e. SEQ ID NOS: 5, 143, and 160) are stimulatory (and/or proliferative) at all concentrations through 500 μg/ml.

The Alamar Blue stain used in the protocol permeates both cell and nuclear membranes, and is metabolized in the mitochondria to cause the change in color. The resulting fluorometric response is therefore a result of total mitochondrial activity caused by cell stimulation and/or mitosis (cell proliferation). The increase in values (for treated cells, as a percent of values for untreated cells) with increased incubation time (120 hours vs. 48 hours) may be attributed to increased cell proliferation in addition to stimulation of cell metabolic activity caused by the peptide

Table 8 presents viability data, as percent of untreated positive control, for human leukocytes (white blood cells, "WBC") in the presence of selected FLAK peptides. The

table also shows for each of these peptides its toxicity (LD50 values) to human red blood cells (RBC) and to human fibroblast cells (WI38). Those certain peptides which are stimulatory to WBCs at low peptide concentrations (i.e. 10 μg/ml or less) and are inhibitory or toxic to WBCs at higher concentrations are also relatively more toxic to RBCs and to fibroblasts than those peptides which are stimulatory and not inhibitory to WBC growth even at concentrations as high as 500 μg/ml.

In limited experiments with other than the Alamar Blue protocol described above, it has been qualitatively determined that those peptides which cause stimulation and proliferation of leukocytes are active upon both the phagocytic and lyphocyte cell components of the mammalian lymphatic system. As such, certain of the stimulatory FLAK peptides which are relatively non-toxic to mammalian cells at therapeutic dose levels may be used as immunomodulators to treat humans or other mammals with compromised immune systems. Such treatment may be administered systemically *in vivo* or by extra-corporeal treatment of whole blood or blood components to be reinfused to the donor. Such therapy would serve to counteract immune deficiency in neutropenic patients caused by age, disease, or chemotherapy and would stimulate natural immune responses to prevent or combat pathogenic infections and growth of certain cancer cell lines or to enhance wound healing processes involving the lymphoid system.Table 9 is a more detailed example (with one peptide, SEQ ID NO:10) of the phenomenon showing the relationships of concentration and time as they effect stimulation, proliferation, and inhibition of the leukocytes.

Table 8: Human leukocyte (WBC) stimulation / proliferation & inhibition by selected FLAK peptides

| | Peptide conc. | 0.1 μg/ml | 0.1 μg/ml | 1 μg/ml | 1 μg/ml | 10 μg/ml | 10 μg/ml |
|---|---|---|---|---|---|---|---|
| SEQ ID NO: | P Number | 48 hours | 120 hours | 48 hours | 120 hours | 48 hours | 120 hours |
| 5 | 12 | 111 | 124 | 115 | 136 | 118 | 141 |
| 10 | 25 | 117 | 135 | 104 | 118 | 99 | 119 |
| 12 | 27 | 108 | 117 | 110 | 126 | 99 | 114 |
| 17 | 35 | 115 | 113 | 119 | 105 | 114 | 81 |
| 20 | 38 | 115 | 110 | 119 | 117 | 114 | 109 |
| 25 | 43 | 115 | 100 | 119 | 114 | 114 | 104 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 58 | 92 | 112 | 120 | 112 | 114 | 98 | 99 |
| 66 | 102 | 100 | 89 | 102 | 90 | 97 | 110 |
| 143 | 182 | 101 | 134 | 96 | 117 | 101 | 133 |
| 150 | 306 | 97 | 94 | 101 | 113 | 94 | 109 |

| SEQ ID NO: | Peptide conc. P Number | 100 µg/ml 48 hours | 100 µg/ml 120 hours | 500 µg/ml 48 hours | 500 µg/ml 120 hours | RBC toxicity LD50 | WI-38 toxicity LD50 |
|---|---|---|---|---|---|---|---|
| 5 | 12 | 116 | 151 | 101 | 119 | >1000 | 950 |
| 10 | 25 | 27 | 43 | 27 | 45 | 60 | 24 |
| 12 | 27 | 30 | 43 | 23 | 39 | 125 | 37 |
| 17 | 35 | 73 | 42 | 72 | 43 | 200 | 8 |
| 20 | 38 | 73 | 60 | 72 | 57 | 350 | 12 |
| 25 | 43 | 73 | 39 | 72 | 37 | 20 | 5 |
| 58 | 92 | 35 | 30 | 26 | 26 | 300 | 125 |
| 66 | 102 | 37 | 32 | 17 | 15 | 300 | 73 |
| 143 | 182 | 109 | 150 | 105 | 132 | >1000 | 660 |
| 150 | 306 | 109 | 140 | 112 | 140 | >1000 | 320 |

Table 9: Human leukocyte (WBC) stimulation / proliferation and inhibition by FLAK peptide SEQ ID NO:10 (P25)

| Time of incubation | 0.1 µg/ml | 1 µg/ml | 10 µg/ml | 100 µg/ml | 500 µg/ml |
|---|---|---|---|---|---|
| 24 hours | 111 | 98 | 88 | 10 | 10 |
| 48 hours | 117 | 104 | 99 | 27 | 27 |
| 72 hours | 119 | 105 | 102 | 31 | 32 |
| 96 hours | 128 | 112 | 110 | 38 | 40 |
| 120 hours | 135 | 118 | 119 | 43 | 45 |

Note: Number values are percent cell viability relative to control cells.

Example 4: Stimulation and proliferation of fibroblasts

The cyQUANT cell proliferation assay provides a convenient, rapid and sensitive procedure for determining the density of cells in culture. The assay has a linear detection range extending from 50 or fewer to at least 50,000 cells in 200 µl volumes using a single dye concentration. The assay is ideal for cell proliferation studies as well as for routine cell counts and can be used to monitor the adherence of cells to surfaces.

Procedure: Different cell lines were maintained with different medium according to the ATCC. Cells were trypsinized with 8 ml of Trypsin (0.25%, Fisher, Pittsburgh, PA). The cell suspension was centrifuged for 10 minutes at 100 rpm. The supernatant was removed and discarded without disturbing the cell pellet. A concentrated cell suspension was prepared in 1.0 ml of medium to obtain a density of about $10^5$ to $10^6$ cells/ml. The actual cell density was determined by counting the cells using a hemocytometer with the Trypan Blue method. Cell numbers were adjusted to obtain equal number of cells per 200 µl volume. Cells were plated with 0% FBS, 2.5% FBS, 5% FBS and 10% FBS. The plates were incubated at 37 °C for a time sufficient to allow the cells to attach. For long-term proliferation studies, 100 µl of medium was removed from each well each day and replaced with fresh medium.

At the desired time, the medium was removed from the adherent cells in a 96 well plate. These cells were already treated with test agents. The cells were frozen in the plate at -70 °C for 30 minutes. The cells were thawed at room temperature. CyQuant GR dry/Cell Lysis Buffer (200 µl) was added to each sample cell. The cells were incubated at room temperature for 15 minutes while protected from the light. Fluorescence was measured using fmax at 485-538 nm.

The above CyQuant protocol was used to examine possible peptide stimulation of fibroblasts. In the following Table 10, data are shown for selected peptides demonstrating their effect on human fibroblast cells (WI38). In the table, the substantial stimulatory and/or proliferative property of selected peptides, as a function of concentration is evident. The values are viability of treated cells expressed as percent (%) above or below positive control (untreated cells). Table 11 shows that the fibroblast stimulation and/or proliferation effect is enhanced for certain peptides in the presence of other growth factors. This is shown by the addition of Fetal Bovine Serum (FBS) to the medium. Negative values indicate inhibitory action of the peptide, especially at concentrations above 10 µg/ml.

Table 10: Human fibroblast (WI-38) cell stimulation by selected FLAK peptides

| SEQ ID NO: | P Number | % FBS in serum | Peptide concentration 0.1 µg/ml | 1 µg/ml | 10 µg/ml | 100 µg/ml |
|---|---|---|---|---|---|---|
| 2 | 2 | 0 | -27 | -3 | 27 | -82 |
| | | 2.5 | 26 | 57 | 23 | -66 |
| 4 | 11 | 0 | 19 | 34 | 50 | -40 |
| | | 2.5 | 50 | 52 | 62 | 14 |
| 6 | 13 | 0 | 76 | 68 | 93 | 95 |
| 8 | 23 | 0 | 21 | 78 | 10 | -48 |
| | | 2.5 | 16 | 23 | 58 | 75 |
| 10 | 25 | 0 | 50 | 59 | 29 | -27 |
| 14 | 27C | 0 | 60 | 85 | 90 | 63 |
| 15 | 30 | 0 | 60 | 75 | 20 | 35 |
| 17 | 35 | 0 | 45 | 70 | 65 | 50 |
| 20 | 38 | 0 | 44 | 22 | 75 | 53 |
| 35 | 55 | 0 | 1 | 12 | 30 | 76 |
| 5 | 12 | 0 (24h inc) | 93 | 90 | 116 | 65 |
| 58 | 92 | 0 (24h inc) | 109 | 114 | 132 | 36 |
| 71 | 107 | 0 | 18 | 27 | 26 | 24 |
| 80 | 118 | 0 | 12 | -4 | -7 | -1 |
| | | 0 (24h inc) | 24 | 55 | 48 | 24 |
| | | 3 | 61 | 70 | 68 | 72 |
| 126 | 165 | 0 | 51 | 77 | 115 | 50 |

Note: Number values are percent cell viability above or below control. Incubations were 48 hours unless otherwise indicated. SEQ ID NOS:5 and 71 are not FLAK peptides.

Table 11: Effect of growth factors on human fibroblast (WI38) cell stimulation

| SEQ ID NO: | P Number | % FBS in serum | Peptide concentration 0.1 µg/ml | 1 µg/ml | 10 µg/ml | 100 µg/ml |
|---|---|---|---|---|---|---|
| 2 | 2 | 0 | -27 | -3 | 27 | -82 |
| | | 2.5 | 26 | 57 | 23 | -66 |
| 4 | 11 | 0 | 19 | 34 | 50 | -40 |
| | | 2.5 | 50 | 52 | 62 | 14 |
| 8 | 23 | 0 | 21 | 78 | 10 | -48 |
| | | 2.5 | 16 | 23 | 58 | 75 |
| 80 | 118 | 0 | 12 | -4 | -7 | -1 |
| | | 3 | 61 | 70 | 68 | 72 |

Note: Number values are percent cell viability above or below control.

Example 5: Toxicity assay - Red blood cell (RBC) hemolysis, and leukocyte (WBC) and fibroblast (WI38) inhibition

Table 12 below summarizes the RBC, WBC, and WI38 toxicity data for typical FLAK peptides. The three RBC, WBC, and WI38 values (LD50) are generally consistent directional indicators of peptide toxicity. In choosing a peptide for possible treatment of a given indication it is important to match the therapeutic activity and specificity of the peptide with its possible toxic properties. The SEQ ID NO:5 peptide is not a FLAK peptide, but rather it is SB-37, a close homolog of Cecropin B. It has previously been shown not to be as active as the FLAK peptides as an antibacterial agent, but to possess wound healing properties as demonstrated *in vivo* in a rat model. This probably results from its stimulatory and proliferative effects on both mammalian leukocytes and fibroblasts.

The protocols for WBC and WI38 stimulation have been discussed above. The RBC protocol follows Table 12.

Table 12: *In vitro* toxicity of selected FLAK peptides on red blood cells (RBC), human leukocytes (WBC), and human fibroblasts (WI38)

| SEQ ID NO: | P Number | RBC LD50 µg/ml | WBC LD50 µg/ml | WI38 LD50 µg/ml |
|---|---|---|---|---|
| 5 | 12 | >1000 | >500 | 60 |
| 10 | 25 | 60 | 79 | 60 |
| 11 | 26 | 900 | 185 | 100 |
| 12 | 27 | 125 | 78 | 60 |
| 16 | 34 | 200 | 77 | 200 |
| 17 | 35 | 200 | 64 | 25 |
| 20 | 38 | 350 | 160 | 100 |
| 25 | 43 | 20 | 70 | 25 |
| 30 | 48 | 130 | 78 | 70 |
| 35 | 55 | 30 | 80 | 28 |
| 58 | 92 | 300 | 51 | 400 |
| 66 | 102 | 300 | 115 | 45 |

The RBC protocol is as follows. Well positions of each dilution and untreated controls are recorded on the lid of a 96-well plate. When the cells were confluent, the media is removed, and replaced with freshly prepared sample dilutions to a final volume of 200 µl. Test agent was added into designed wells of the 96-well plate. The 200 µl

fresh medium was added to positive control wells; and 200 µl of 70% ethanol was added to negative control wells. The plate was incubated overnight at 37 °C, 5% $CO_2$, and at least 90% humidity. Room temperature AlamarBlue solution (20 µl) was added to all wells. The plates were read spectrofluorometrically (excitation 544 nm, emission 590 nm). The plates were incubated for 3 hours at 37 °C, 5% $CO_2$, and at least 90% humidity. The plates were read again at 3 and 24 hours incubation. The LD50 endpoint was determined from the graph by reading from where the 50 percent point intercepts the Dose Response Curve to the concentration along the x-axis. That concentration is the LD50 value. The LD50 value for test agents within a single test agent class can be used to rank-order their relative toxicities or to correlate with *in vivo* data.

This hemolytic assay is based upon that presented in *Journal of Peptide Research* 53: 82-90 (1999). Preparation of all media, stock solutions and dilutions were performed in a laminar flow hood to minimize or prevent contamination. All procedures were performed according to safety protocols pertaining to the handling and disposal of human body fluids.

Red blood cells (RBCs) were washed three times with PBS (35 mM phosphate buffer 0.15 M NaCl, pH 7.0). RBCs suspended in PBS (0.4% (v/v); about 10 ml per 15 peptides) were prepared. Suspensions (100 µl) were aliquoted to each sample and control tube. Serially diluted peptide solutions (100 µl) were pipetted into the sample tubes. Negative control tubes contained 100 µl PBS; positive control tubes contained 100 µl 1% Triton-X100 detergent. All tubes were incubated for 1 hour at 37 °C. The tubes were removed from the incubator and centrifuged at 1000g for 5 minutes. Supernatant (100 µl) was pipetted to a 96-well polyvinyl chloride plate. The absorbance at 414 nm ($A_{414}$) was measured, and used to calculate the percent hemolysis according to the following formula.

$$\frac{(A_{414} \text{ in peptide solution} - A_{414} \text{ in PBS})}{(A_{414} \text{ in Triton-X 100} - A_{414} \text{ in PBS})} \times 100\%$$

Percent hemolysis is plotted against peptide concentration, and the concentration at which 50% hemolysis is determined ($LD_{50}$). The following Table 13 details the results of the hemolytic assay using the peptides discussed herein.

Table 13

| Peptide name | SEQ ID NO: | P Number | $LD_{50}$ µg/mL |
|---|---|---|---|
| Hecate AC #1010 | 1 | 1 | 100 |
| Hecate AM | 2 | 2 | 10 |
| SB-37 AC #1018 | 3 | 5 | > |
| Shiva 10 AM | 4 | 11 | 76 |
| SB-37 AM | 5 | 12 | > |
| Shiva 10 AC #1015 | 6 | 13 | 50 |
| Magainin 2 | 7 | 16 | 550 |
| FLAK01 AM | 8 | 23 | 300 |
| FLAK03 AM | 9 | 24 | 10 |
| FLAK04 AM | 10 | 25 | 16 |
| FLAK05 AM | 11 | 26 | 90 |
| FLAK06 AM | 12 | 27 | 125 |
| FLAK06 AC | 13 | 27B | 700 |
| FLAK06 R-AC | 14 | 27C | 250 |
| KALV | 15 | 30 | 150 |
| FLAK 17 AM | 16 | 34 | 200 |
| FLAK 26 AM | 17 | 35 | 200 |
| FLAK 25 AM | 18 | 36 | 85 |
| Hecate 2DAc | 19 | 37 | 30 |
| FLAK43 AM | 20 | 38 | 350 |
| FLAK44 AM | 21 | 39 | > |
| FLAK62 AM | 22 | 40 | > |
| FLAK 06R-AM | 23 | 41 | 40 |
| MSI-78 AM | 24 | 42 | 300 |
| FLAK50 | 25 | 43 | 20 |
| FLAK51 | 26 | 44 | 90 |
| FLAK57 | 27 | 45 | 700 |
| FLAK71 | 28 | 46 | 900 |
| FLAK77 | 29 | 47 | > |
| FLAK50V | 30 | 48 | 200 |
| FLAK50F | 31 | 49 | 225 |
| FLAK26V AM | 32 | 50 | 420 |
| CAME-15 | 33 | 53 | 20 |
| FLAK50C | 34 | 54 | 250 |
| FLAK50D | 35 | 55 | 20 |
| FLAK 50E | 36 | 56 | 600 |
| FLAK80 | 37 | 57 | > |

| | | | |
|---|---|---|---|
| FLAK81 | 38 | 58 | > |
| FLAK82 | 39 | 59 | 1000 |
| FLAK83M | 40 | 60 | > |
| FLAK 26 Ac | 41 | 61 | 390 |
| Indolicidin | 42 | 63 | 375 |
| FLAK 17 C | 43 | 64 | 6 |
| FLAK 50H | 44 | 65 | 950 |
| FLAK 50G | 45 | 66 | 600 |
| Shiva deriv P69+KWKL | 46 | 70 | 80 |
| Shiva 10 (1-18_AC | 47 | 71 | > |
| Shiva 10 peptide 71+KWKL | 48 | 72 | 110 |
| CA(1-7)Shiva10(1-16) | 49 | 73 | 90 |
| FLAK 54 | 50 | 74 | > |
| FLAK 56 | 51 | 75 | 750 |
| FLAK 58 | 52 | 76 | > |
| FLAK 72 | 53 | 77 | > |
| FLAK 75 | 54 | 79 | > |
| Shiva 10 (1-16) Ac | 55 | 80 | 900 |
| CA(1-7)Shiva10(1-16)-COOH | 56 | 81 | 8 |
| Indolocidin-ac | 57 | 91 | 40 |
| FLAK50B | 58 | 92 | 300 |
| FLAK50J | 59 | 93 | > |
| FLAK50I | 60 | 94 | 350 |
| FLAK50K | 61 | 95 | > |
| FLAK50L | 62 | 96 | > |
| Shiva-11 | 63 | 98 | 60 |
| Shiva 11[(1-16)ME(2-9)]-COOH | 64 | 99 | 25 |
| FLAK 50N | 65 | 101 | 550 |
| FLAK 50O | 66 | 102 | 500 |
| FLAK 50P | 67 | 103 | 650 |
| CA(1-&Hecate(11/23) | 68 | 104 | 70 |
| PYL-ME | 69 | 105 | ND |
| FLAG26-D1 | 70 | 106 | > |
| Vishnu3 | 71 | 107 | > |
| Melittin | 72 | 108 | <1 |
| FLAK26-D2 | 73 | 109 | > |
| FLAG26-D3 | 74 | 110 | > |
| FLAK50 Q1 | 75 | 111 | 60 |
| FLAK50 Q2 | 76 | 112 | > |
| FLAK50 Q3 | 77 | 113 | 1000 |
| FLAK50 Q4 | 78 | 114 | > |
| FLAK50 Q5 | 79 | 117 | > |
| FLAK50 Q6 | 80 | 118 | 700 |

| | | | |
|---|---|---|---|
| FLAK50 Q7 | 81 | 119 | 400 |
| FLAK50 Q8 | 82 | 120 | > |
| FLAK50 Q9 | 83 | 121 | > |
| FLAK50 Q10 | 84 | 122 | > |
| FLAK50 T1 | 85 | 123 | 1000 |
| FLAK50 T2 | 86 | 124 | 55 |
| FLAK50 T3 | 87 | 125 | > |
| FLAK50 T4 | 88 | 126 | > |
| FLAK50 T5 | 89 | 127 | > |
| FLAK90 | 90 | 128 | > |
| FLAK91 | 91 | 129 | > |
| FLAK92 | 92 | 130 | > |
| FLAK93 | 93 | 131 | > |
| FLAK50 Z1 | 94 | 132 | > |
| FLAK50 Z2 | 95 | 133 | > |
| FLAK50 Z3 | 96 | 134 | > |
| FLAK50 Z4 | 97 | 135 | 900 |
| FLAK50 Z5 | 98 | 136 | > |
| FLAK50 Z6 | 99 | 137 | > |
| FLAK50 Z7 | 100 | 138 | 20 |
| FLAK50 Z8 | 101 | 139 | > |
| FLAK50 Z9 | 102 | 140 | > |
| FLAK94 | 103 | 141 | 900 |
| FLAK93B | 104 | 142 | 900 |
| FLAK50 Z10 | 105 | 143 | > |
| FLAK96 | 106 | 144 | 600 |
| FLAK97 | 107 | 145 | > |
| FLAK98 | 108 | 146 | 180 |
| FKRLA | 109 | 147 | 300 |
| FLAK91B | 110 | 148 | > |
| FLAK92B | 111 | 149 | > |
| FLAK99 | 112 | 150 | 650 |
| FLAK50T6 | 113 | 151 | > |
| FLAK50T7 | 114 | 152 | 880 |
| FLAK95 | 115 | 153 | 800 |
| FLAK50T8 | 116 | 154 | 450 |
| FLAK50T9 | 117 | 155 | > |
| FLAK100-CO2H | 118 | 156 | 10 |
| FAGVL | 119 | 157 | 850 |
| Modelin-5 | 120 | 159 | ND |
| Modelin-5-CO2H | 121 | 160 | > |
| FLAK120 | 126 | 165 | 350 |
| FLAK121 | 127 | 166 | > |
| FLAK96B | 128 | 167 | 200 |

| | | | |
|---|---|---|---|
| FLAK96G | 129 | 168 | 600 |
| FLAK96F | 130 | 169 | > |
| FLAK96C | 131 | 170 | > |
| FLAK96D | 132 | 171 | 550 |
| Modelin-8D | 135 | 174 | > |
| Modelin-8E | 136 | 175 | > |
| Flak 96 | 137 | 176 | > |
| Flak 96I | 138 | 177 | 400 |
| Flak 96J | 139 | 178 | > |
| Flak 96L | 140 | 179 | 850 |
| FLAK-120G | 141 | 180 | > |
| FLAK-120D | 142 | 181 | > |
| FLAK-120C | 143 | 182 | > |
| FLAK-120B | 144 | 183 | > |
| FLAK-120F | 145 | 184 | 850 |
| Magainin2wisc | 146 | 300 | 250 |
| D2A21 | 147 | 301 | 10 |
| KSL-1 | 148 | 302 | > |
| KSL-7 | 149 | 303 | 500 |
| LSB-37 | 150 | 306 | > |
| Anubis-2 | 151 | 307 | > |
| FLAK17CV | 152 | 501 | 15 |
| FLAK50Q1V | 153 | 502 | 100 |
| D2A21V | 154 | 503 | 20 |
| FLAK25AMV | 155 | 504 | 70 |
| FLAK43AMV | 156 | 505 | 620 |
| FLAK50DV | 157 | 506 | 120 |
| HECATE AMV | 158 | 507 | 20 |
| HECATE ACV | 159 | 508 | 70 |
| FLAK04AMV | 160 | 509 | 40 |
| FLAK03AMV | 161 | 510 | 10 |
| D-Shiva 10 AC | 162 | 67 | 40 |
| Shiva 11 AC | 163 | 100 | > |
| Shiva 10 (1-18)AM | 164 | 69 | 900 |

Note: > indicates greater than 1000; ND = not determined.

Example 6: Effects of valine substitution

Changing a peptide sequence where the first amino acid is valine, and particularly when the first amino acid is changed from phenylalanine to valine, can lead to desirable properties. The red blood cell and fibroblast cell (WI38) toxicity can be decreased, while not significantly decreasing other desirable properties. Table 14 below shows numerous

examples (14) of reducing the indicated toxicity of a peptide as seen from increase in viability of both red blood cells and fibroblast cells when treated with peptide. LD50 values are in μg/ml.

Table 14

| SEQ. ID NO: | P No. | Sequence | Hemolysis RBC LD50 | WI-38 LD50 |
|---|---|---|---|---|
| 2 | 2 | FALALKALKKALKKLKKALKKAL-NH2 | 12 | 66 |
| 15 | 30 | VALALKALKKALKKLKKALKKAL-NH2 | 150 | 93 |
| | | | | |
| 17 | 35 | FAKKLAKLAKKLAKLAL-NH2 | 150 | 25 |
| 32 | 50 | VAKKLAKLAKKLAKLAL-NH2 | 420 | 45 |
| | | | | |
| 25 | 43 | FAKLLAKLAKKLL-NH2 | 20 | 25 |
| 30 | 48 | VAKLLAKLAKKLL-NH2 | 130 | 160 |
| | | | | |
| 86 | 124 | FAKLLAKLAKKVL-NH2 | 55 | 21 |
| 116 | 154 | VAKLLAKLAKKVL-NH2 | 870 | 110 |
| | | | | |
| 126 | 165 | FALALKALKKL-NH2 | 350 | 850 |
| 141 | 180 | VALALKALKKL-NH2 | 850 | 1000 |
| | | | | |
| 43 | 64 | FAKALKALLKALKAL-NH2 | 6 | 37 |
| 152 | 501 | VAKALKALLKALKAL-NH2 | 15 | 26 |
| | | | | |
| 75 | 111 | FAKFLAKFLKKAL-NH2 | 5 | 25 |
| 153 | 502 | VAKFLAKFLKKAL-NH2 | 100 | 64 |
| | | | | |
| 147 | 301 | FAKKFAKKFKKFAKKFAKFAFAF-NH2 | 10 | 66 |
| 154 | 503 | VAKKFAKKFKKFAKKFAKFAFAF-NH2 | 20 | 150 |
| | | | | |
| 18 | 36 | FAKKLAKLAKKLAKLALAL-NH2 | 12 | 19 |
| 155 | 504 | VAKKLAKLAKKLAKLALAL-NH2 | 70 | 110 |
| | | | | |
| 20 | 38 | FAKKLAKLAKKLLAL-NH2 | 350 | 100 |
| 156 | 505 | VAKKLAKLAKKLLAL-NH2 | 620 | 85 |
| | | | | |
| 35 | 55 | FAKLLAKALKKLL-NH2 | 20 | 32 |
| 157 | 506 | VAKLLAKALKKLL-NH2 | 120 | 75 |
| | | | | |
| 1 | 1 | FALALKALKKALKKLKKALKKAL-COOH | 20 | 27 |
| 159 | 508 | VALALKALKKALKKLKKALKKAL-COOH | 70 | 190 |
| | | | | |

| | | | | |
|---|---|---|---|---|
| 10 | 25 | FALALKALKKLAKKLKKLAKKAL-NH2 | 16 | 24 |
| 160 | 509 | VALALKALKKLAKKLKKLAKKAL-NH2 | 40 | 95 |
| | | | | |
| 9 | 24 | FALALKALKKLLKKLKKLAKKAL-NH2 | 10 | 55 |
| 161 | 510 | VALALKALKKLLKKLKKLAKKAL-NH2 | 10 | 77 |

Although the effects of reduction of toxicity to mammalian cells by valine substitution is accompanied by modest reductions of therapeutic activity against microbial pathogens and cancer cells, there are some cases in which the valine substitution results in a desirable increase in therapeutic activity. This can be seen in the following Table 15 where it is shown that the valine substitution in some cases has increased the peptide's activity against the gram negative bacterium *Pseudomonas*.

Hemolysis and WI38 values represent LD50 values. P. aerug values represent MIC values in μg/mL against *Pseudomonas aeruginosa* ATCC accession number 9027.

Table 15

| SEQ ID NO: | P No. | Sequence | Hemolysis | WI38 | P. aerug |
|---|---|---|---|---|---|
| 17 | 35 | FAKKLAKLAKKLAKLAL | 100 | 25 | 200 |
| 32 | 50 | VAKKLAKLAKKLAKLAL | 420 | 45 | 15 |
| | | | | | |
| 25 | 43 | FAKLLAKLAKKLL | 20 | 25 | 100 |
| 30 | 48 | VAKLLAKLAKKLL | 200 | 160 | 5 |
| | | | | | |
| 86 | 124 | FAKLLAKLAKKVL | 300 | 21 | 100 |
| 116 | 154 | VAKLLAKLAKKVL | 450 | 110 | 100 |

Example 7: Preferred peptides

Preferred peptides can be selected from the above described experimental data. Preferred antimicrobial peptides for gram positive or gram negative bacteria can be selected as having MIC values of less than or equal to about 10 μg/ml, or as having MBC values of less than or equal to about 25 μg/ml. Preferred antifungal peptides can be selected as having MIC or MBC values of less than or equal to about 25 μg/ml. Preferred anticancer peptides can be selected as having LD50 values of less than or equal to about 25 μg/ml.

The following Table 16 lists representative preferred peptides, where an 'X' indicates that the peptide is a preferred peptide for that column's property. The peptide's "length" is the number of amino acid residues in the sequence.

Table 16

| SEQ ID NO: | P-number | Length (AA) | Anti-bacterial | Anti-fungal | Anti-cancer |
|---|---|---|---|---|---|
| 1 | 1 | 23 | X | | X |
| 2 | 2 | 23 | X | X | X |
| 4 | 11 | 23 | X | | |
| 6 | 13 | 23 | X | | |
| 8 | 23 | 23 | X | | X |
| 10 | 25 | 23 | X | X | |
| 11 | 26 | 21 | X | X | X |
| 12 | 27 | 19 | X | X | |
| 13 | 27B | 19 | X | | |
| 14 | 27C | 19 | X | | |
| 15 | 30 | 23 | X | X | X |
| 16 | 34 | 16 | X | X | X |
| 17 | 35 | 17 | X | | X |
| 18 | 36 | 19 | X | | X |
| 19 | 37 | 23 | X | | X |
| 20 | 38 | 15 | X | | |
| 23 | 41 | 19 | X | X | X |
| 25 | 43 | 13 | X | | X |
| 26 | 44 | 15 | X | | |
| 27 | 45 | 14 | X | | |
| 28 | 46 | 15 | X | | X |
| 29 | 47 | 12 | | X | X |
| 30 | 48 | 13 | X | | |
| 31 | 49 | 12 | X | | X |
| 32 | 50 | 17 | X | | |
| 34 | 54 | 13 | X | X | X |
| 35 | 55 | 13 | X | | |
| 36 | 56 | 13 | X | | |
| 41 | 61 | 15 | X | | |
| 43 | 64 | 15 | X | | |
| 45 | 66 | 13 | X | | X |
| 46 | 70 | 23 | X | | |
| 50 | 74 | 13 | X | | X |
| 51 | 75 | 13 | X | | |
| 52 | 76 | 14 | X | | |

| | | | | | |
|---|---|---|---|---|---|
| 55 | 80 | 23 | X | | X |
| 56 | 81 | 23 | X | | X |
| 57 | 91 | 15 | X | X | X |
| 58 | 92 | 13 | X | | X |
| 60 | 94 | 13 | X | | |
| 65 | 101 | 13 | X | X | |
| 66 | 102 | 13 | X | X | |
| 67 | 103 | 12 | X | | X |
| 68 | 104 | 20 | X | | |
| 74 | 110 | 12 | X | | X |
| 75 | 111 | 13 | X | | |
| 77 | 113 | 13 | X | X | |
| 80 | 118 | 13 | X | X | |
| 81 | 119 | 14 | X | X | |
| 84 | 122 | 13 | X | X | |
| 85 | 123 | 10 | | X | X |
| 86 | 124 | 13 | X | | |
| 87 | 125 | 13 | X | | |
| 93 | 131 | 5 | X | X | |
| 106 | 144 | 12 | X | X | |
| 108 | 146 | 13 | X | | |
| 112 | 150 | 17 | X | X | |
| 115 | 153 | 17 | | X | |
| 116 | 154 | 13 | X | X | |
| 126 | 165 | 11 | X | X | |
| 128 | 167 | 12 | | X | |
| 131 | 170 | 10 | | X | |
| 143 | 182 | 10 | | | X |
| 152 | 501 | 15 | X | | X |
| 162 | 67 | 23 | X | X | |
| 163 | 100 | 13 | X | | |
| 164 | 69 | 23 | X | X | |
| 165 | 97 | 13 | | | |

Preferred peptides for stimulation and proliferation can also be selected. The following Table 17 lists representative preferred peptides, where an 'X' indicates that the peptide is a preferred peptide for that column's property. Peptides which are stimulatory for leukocytes at 0.1 μg/ml to 1.0 μg/ml concentration are preferred, as at this concentration the peptides are not toxic to red blood cells, WI-38 fibroblasts, or to human leukocytes. Peptides which are stimulatory for fibroblasts at 0.1 μg/ml to 1.0 μg/ml are preferred, as at this concentration the peptides are not toxic.

Table 17: Preferred peptides for leukocyte and fibroblast stimulation / proliferation

| SEQ ID NO: | P-number | Length | Leukocyte | Fibroblast |
|---|---|---|---|---|
| 1 | 29 | 23 | X | X |
| 2 | 2 | 23 | X | X |
| 5 | 12 | 38 | X | X |
| 6 | 13 | 23 | X | X |
| 8 | 23 | 23 | X | X |
| 10 | 25 | 23 | X | X |
| 11 | 26 | 21 | X | X |
| 12 | 27 | 19 | X | X |
| 13 | 27B | 19 | X | X |
| 14 | 27C | 19 | X | X |
| 15 | 30 | 23 | X | X |
| 16 | 34 | 16 | X | X |
| 17 | 35 | 17 | | X |
| 20 | 38 | 15 | | X |
| 27 | 45 | 14 | | X |
| 28 | 46 | 15 | | X |
| 30 | 48 | 13 | | X |
| 32 | 50 | 17 | | |
| 34 | 54 | 13 | X | X |
| 45 | 66 | 13 | X | X |
| 46 | 70 | 23 | X | X |
| 50 | 74 | 13 | X | X |
| 51 | 75 | 13 | | X |
| 55 | 80 | 23 | | X |
| 56 | 81 | 23 | | X |
| 57 | 91 | 15 | X | X |
| 58 | 92 | 13 | X | X |
| 59 | 93 | 13 | | X |
| 60 | 94 | 13 | | X |
| 61 | 95 | 13 | X | X |
| 65 | 101 | 13 | | X |
| 66 | 102 | 13 | | X |
| 71 | 107 | 19 | X | X |
| 74 | 110 | 12 | | X |
| 75 | 111 | 13 | | X |
| 77 | 113 | 13 | | X |
| 80 | 118 | 13 | | X |
| 81 | 119 | 14 | | X |
| 87 | 125 | 13 | X | X |
| 90 | 128 | 5 | X | X |

| | | | | |
|---|---|---|---|---|
| 91 | 129 | 5 | | X |
| 92 | 130 | 5 | | X |
| 115 | 153 | 17 | | X |
| 116 | 154 | 13 | X | |
| 126 | 165 | 11 | | X |
| 127 | 166 | 11 | | X |
| 129 | 168 | 6 | X | X |
| 132 | 171 | 11 | | X |
| 137 | 176 | 11 | X | |
| 138 | 177 | 12 | X | |
| 139 | 178 | 11 | X | X |
| 140 | 179 | 11 | X | X |
| 141 | 180 | 11 | X | X |
| 142 | 181 | 10 | X | X |
| 143 | 182 | 10 | X | X |
| 144 | 183 | 5 | X | X |
| 145 | 184 | 5 | X | X |
| 159 | 508 | 23 | X | X |
| 162 | 67 | 23 | X | X |
| 164 | 69 | 18 | | X |

Example 8: Synergistic effects with lysozyme

Synergy between lytic peptides and lysozyme was assayed. Sterilized milk was inoculated with bacteria to 5 x $10^5$ per ml. Peptide Shiva-10 (SEQ ID NO:4) was added to 10 μg/ml, and chicken lysozyme was added to 1 mg/ml. The percent killing of bacteria was determined.

Table 18

| | *Staph. aureus* | *Pseud. aeruginosa* |
|---|---|---|
| Peptide and lysozyme | 0% | 100% |
| Peptide | 0% | 0% |
| Lysozyme | 0% | 0% |

Synergy between cecropin SB-37 (SEQ ID NO:5) and lysozyme was determined against *Pseudomonas syringae* pv. *tabaci* (PSPT), *Pseudomonas solanacearum* (PS), *Erwinia caratovora subsp. carotova* (EC), and *Xanthomonas campestris* pv. *campestris* (XC). $LD_{50}$ (μM) values were determined.

Table 19

| | SB-37 | Lysozyme | SB-37 and Lysozyme |
|---|---|---|---|
| PSPT | 5.20 | > | 0.19 |
| PS | 64.0 | > | 16.0 |
| EC | 1.48 | > | 0.44 |
| XC | 0.57 | > | 0.027 |

> indicates greater than 1000.

Synergy between Shiva-1 and lysozyme was determined. The percent viability of *Pseudomonas aeruginosa* was determined relative to blank controls. Lysozyme was used at the same molar concentration as the peptide.

Table 20

| Peptide concentration (μM) | SB-37 | Shiva-1 | Lysozyme (1x) | Shiva-1 and Lysozyme (1x) |
|---|---|---|---|---|
| 0 | 100 | 100 | 100 | 100 |
| 0.01 | 100 | 100 | 100 | 56.6 |
| 0.1 | 79.4 | 69.6 | 82.2 | 25.8 |
| 1 | 48.8 | 37.9 | 52.1 | 4.4 |
| 5 | 38.5 | 1.5 | 7.9 | 0.2 |
| 7.5 | 0.7 | 0.1 | 0.6 | 0 |
| 25 | 0 | 0 | 0.4 | 0 |

Synergy between Shiva-1 and lysozyme was determined. The percent viability of gram positive *S. intermedius* 19930, *S. intermedius* 20034, and *S. aureus* was determined relative to blank controls. Lysozyme was used at ten times the molar concentration as the peptide.

Table 21: *S. intermedius* 19930

| Peptide concentration (μM) | SB-37 | Shiva-1 | Lysozyme (10x) | Shiva-1 and Lysozyme (10x) |
|---|---|---|---|---|
| 0 | 100 | 100 | 100 | 100 |
| 0.01 | 100 | 100 | 100 | 100 |
| 0.1 | 94.7 | 81.8 | 100 | 79.2 |
| 0.5 | 69.4 | 65.0 | 81.3 | 65.1 |
| 1 | 42.5 | 42.1 | 53 | 43 |
| 5 | 36.1 | 35.2 | 49.5 | 17.2 |
| 10 | 5.6 | 1.2 | 34.4 | 1.1 |
| 50 | 0 | 0 | 22 | 0 |

Table 22: *S. intermedius* 20034

| Peptide concentration (μM) | SB-37 | Shiva-1 | Lysozyme (10x) | Shiva-1 and Lysozyme (10x) |
|---|---|---|---|---|
| 0 | 100 | 100 | 100 | 100 |
| 0.01 | 100 | 100 | 100 | 100 |
| 0.25 | 85.4 | 87.1 | 100 | 85.1 |
| 0.5 | 68.0 | 80.0 | 59.0 | 53.4 |
| 0.75 | 62.2 | 60.1 | 42.3 | 41.0 |
| 5 | 35.1 | 4.1 | 38.3 | 4.3 |
| 50 | 0 | 0 | 10.0 | 0 |

Table 23: *S. aureus*

| Peptide concentration (μM) | SB-37 | Shiva-1 | Lysozyme (10x) | Shiva-1 and Lysozyme (10x) |
|---|---|---|---|---|
| 0 | 100 | 100 | 100 | 100 |
| 0.01 | 100 | 100 | 100 | 100 |
| 0.1 | 100 | 100 | 100 | 100 |
| 0.5 | 81.0 | 50.1 | 100 | 100 |
| 1 | 47.5 | 24.4 | 51.0 | 31.2 |
| 5 | 31.8 | 15.9 | 18.4 | 8.2 |
| 10 | 5.6 | 4.5 | 13.3 | 4.5 |
| 50 | 1.9 | 1.6 | 9.5 | 1.4 |

Synergy experiments can also be performed using peptides in the presence of EDTA, which potentiates the peptides additively or synergistically.

Example 9: Synergistic effects with antibiotics

Synergy between peptide Shiva-10 (SEQ ID NO:4) and various antimicrobial agents was investigated against *Escherichia coli* 25922. The following table illustrates the beneficial effects of combining the peptide with the agents, where the numbers are the minimum bactericidal concentration (MBC; μg/mL).

Table 24

| Agent | Without peptide | With peptide |
|---|---|---|
| Shiva-10 | 50 | n/a |
| Ticarcillin | 100 | 50 (15 μg/mL peptide) |
| Cefoperazone | 150 | 2.5 (15 μg/mL peptide) |
| Doxycycline | 5 | 1 (15 μg/mL peptide) |
| Neomycin | 100 | 5 (5 μg/mL peptide) |

| | | |
|---|---|---|
| Amikacin | 150 | 50 (5 μg/mL peptide) |
| Tetracycline | 10 | 2.5 (5 μg/mL peptide) |

Synergy between peptide Shiva-10 (SEQ ID NO:4) and various antimicrobial agents was investigated against *Staph. aureus* 29213. The following table illustrates the beneficial effects of combining the peptide with the agents, where the numbers are the minimum bactericidal concentration (MBC; μg/mL).

Table 25

| Agent | Without peptide | With 5 μg/mL peptide |
|---|---|---|
| Shiva-10 | 200 | n/a |
| Ampicillin | 5 | 2.5 |
| Ticarcillin | 25 | 15 |
| Cefoperazone | 10 | 2.5 |
| Tobramycin | 25 | 10 |
| Tetracycline | 10 | 1 |

Synergy between peptide FLAK 26AM (P35; SEQ ID NO:17) and various antimicrobial agents was investigated against *Staph. aureus* 29213 MBC. The following table illustrates the beneficial effects of combining the peptide with the agents, where the numbers are the minimum bactericidal concentration (MBC; μg/mL). This experiment determined the peptide MBC in the absence of the antimicrobial agent, or in the presence of the indicated concentration of antimicrobial agent

Table 26

| Agent | MBC of peptide |
|---|---|
| FLAK 26AM alone | 50 |
| Vancomycin (1 ppm) | 32 |
| Cefoperazone (0.25 ppm) | 20 |

Synergy between doxacycline and various peptides was investigated against *P. aeruginosa* 27853. The following table illustrates the beneficial effects of combining doxacycline and the peptides, where the numbers are the minimum bactericidal concentration (MBC; μg/mL). When combined with the peptides, the doxacycline was held at 10 ppm concentration.

Table 27

| Agent | Without doxacycline | With doxacycline |
|---|---|---|
| Doxacycline | n/a | 100 |
| SB-37 (P5; SEQ ID NO:3) | 200 | 30 |
| FLAK 26AM (P35; SEQ ID NO:17) | 50 | 32 |

Synergy between tetracycline and various peptides was investigated against *Escherichia coli* 25922 MBC. The following table illustrates the beneficial effects of combining tetracycline and the peptides, where the numbers are the minimum bactericidal concentration (MBC; μg/mL). When combined with the peptides, the concentration of tetracycline was held at 1.5 ppm.

Table 28

| Agent | Without tetracycline | With tetracycline |
|---|---|---|
| Tetracycline | n/a | 10 |
| FLAK 06AM (P27; SEQ ID NO:12) | 75 | 25 |
| FLAK 26AM (P35; SEQ ID NO:17) | 50 | 20 |

Example 10: Synergistic effects with chemotherapy agents

Other investigators have reported that lytic peptides which are inhibitory to cancer cells will act synergistically with conventional cancer chemotherapy drugs. The FLAK peptides are no exception. Table 29 below demonstrates for example that selected FLAK peptides are synergistic with Tamoxifen in the inhibition of the MCF7 line of breast cancer cells. Table 30 lists other more active anti-cancer peptide candidates for synergistic application with Tamoxifen or other cancer therapy drugs.

Tables 29 and 30 also show toxicity of the selected peptides against RBCs, WBCs, and WI38 cells. When used at very low non-toxic levels selected anti-cancer peptides can synergistically potentiate other chemotherapy agents to permit their effective use at substantially lower dose levels with consequently fewer side effects.

Table 29: Synergy of FLAK peptides with tamoxifen on MCF7 cells

| | | Active agent | LD50 on MCF7 cells | | |
|---|---|---|---|---|---|
| SEQ ID NO: (P No.) | Agent | MCF7 LD50 μg/ml | Peptide conc. μg/ml | Tamox. conc. μg/ml | Total conc. μg/ml |
| | Tamoxifen | 20 | 0 | 20 | 20 |

| | | | | | |
|---|---|---|---|---|---|
| 164 (69) | Alone<br>With Tamox. | 79 | 2.5 | 4.6 | 7.1 |
| 145 (184) | Alone<br>With Tamox. | 240 | 10 | 4 | 14 |
| 121 (160) | Alone<br>With Tamox. | 240 | 11 | 3.7 | 14.7 |
| 106 (144) | Alone<br>With Tamox. | 310 | 35 | 7.7 | 42.7 |

| SEQ ID NO: (P No.) | MCF7 LD50 µg/ml | RBC LD50 µg/ml | WI38 LD50 µg/ml | WBC LD50 µg/ml |
|---|---|---|---|---|
| 164 (69) | 79 | 900 | 60 | 140 |
| 145 (184) | 240 | 850 | 1000 | 410 |
| 121 (160) | 240 | > 1000 | 700 | 900 |
| 106 (144) | 310 | 600 | 740 | 320 |
| 17 (35) | 9 | 200 | 25 | 25 |
| 32 (50) | 32 | 420 | 40 | 420 |
| 20 (38) | 17 | 350 | 100 | 54 |

Table 30: Other highly active peptide candidates for synergistic anti-cancer applications

| SEQ ID NO: (P No.) | MCF7 LD50 µg/ml | RBC LD50 µg/ml | WI38 LD50 µg/ml | WBC LD50 µg/ml |
|---|---|---|---|---|
| 17 (35) | 9 | 200 | 25 | 25 |
| 32 (50) | 32 | 420 | 40 | 420 |
| 20 (38) | 17 | 350 | 100 | 54 |

Example 11: Synergistic effects with growth factors

It has been shown above in Example 4 and Table 11 that certain of the FLAK peptides are synergistic with other mitogens or growth factors in the stimulatory and/or proliferative properties of the peptides.

Example 12: Activity against drug resistant strains

Peptides were assayed for their activity against tobramycin sensitive and resistant strains. As shown in the following Table 31, peptides P56 (SEQ ID NO:36), P74 (SEQ ID NO:50), and P125 (SEQ ID NO:87) showed enhanced activity against tobramycin resistant (tr) *Pseudomonas* ATCC 13096 than against tobramycin sensitive (ts)

*Pseudomonas* ATCC 27853. The same three peptides showed enhanced activity against clinical tobramycin resistant strain 960890198-3c (Table 31).

Table 31

| Peptide | tr *Pseudomonas* 13096 | ts *Pseudomonas* 27853 |
|---|---|---|
| SEQ ID NO:36 (P56) | 16 | 125 |
| SEQ ID NO:50 (P74) | 16 | 125 |
| SEQ ID NO:87 (P125) | 4 | 31 |

Table 32

| Peptide | tr *Pseudomonas* 960890198-3c | ts *Pseudomonas* 27853 |
|---|---|---|
| SEQ ID NO:36 (P56) | > 50 | 125 |
| SEQ ID NO:50 (P74) | 25 | 125 |
| SEQ ID NO:87 (P92) | 50 | 63 |

Example 13: Wound healing

The inventive peptides can be used in compositions for topical or systemic delivery in wound healing applications. The compositions can be a liquid, cream, paste, or other pharmaceutically acceptable formulation. The compositions may contain other biologically active agents. The compositions may contain pharmaceutically acceptable carriers.

Those peptides preferred for wound healing, shown in Table 33 below, are peptides which were preferred for either, or or both, leukocyte or fibroblast stimulation.

Table 33: Preferred peptides for wound healing

| SEQ ID NO: | P No. | | SEQ ID NO: | P No. | | SEQ ID NO: | P No. |
|---|---|---|---|---|---|---|---|
| 1 | 1 | | 50 | 74 | | 93 | 131 |
| 2 | 2 | | 51 | 75 | | 115 | 153 |
| 5 | 12 | | 55 | 80 | | 116 | 154 |
| 6 | 13 | | 56 | 81 | | 126 | 165 |
| 8 | 23 | | 57 | 91 | | 127 | 166 |
| 10 | 25 | | 58 | 92 | | 129 | 168 |
| 11 | 26 | | 59 | 93 | | 132 | 171 |
| 12 | 27 | | 60 | 94 | | 137 | 176 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 13 | 27B | | 61 | 95 | | 138 | 177 |
| 14 | 27C | | 65 | 101 | | 139 | 178 |
| 15 | 30 | | 66 | 102 | | 140 | 179 |
| 16 | 34 | | 71 | 107 | | 141 | 180 |
| 17 | 35 | | 74 | 110 | | 142 | 181 |
| 20 | 38 | | 75 | 111 | | 143 | 182 |
| 27 | 45 | | 77 | 113 | | 144 | 183 |
| 28 | 46 | | 80 | 118 | | 145 | 184 |
| 30 | 48 | | 81 | 119 | | 159 | 508 |
| 32 | 50 | | 87 | 125 | | 162 | 67 |
| 34 | 54 | | 90 | 128 | | 164 | 69 |
| 45 | 66 | | 91 | 129 | | | |
| 46 | 70 | | 92 | 130 | | | |

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.